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(54) Title: BIODEGRADABLE AZO DYES

(57) Abstract

A biodegradable azo dye contains a nitrogen atom linked to an aromatic ring having a lignin-like substitution pattern. The ring is preferably a syringyl or guaiacol moiety, and provides a naturally-occurring structure for attack by microorganisms, such as *Streptomyces* or *Phanerochaete*. In especially preferred embodiments, the aromatic ring has a first substituent R₁ selected from among hydroxy, lower alkoxy, or amino, and a second substituent R₂ selected from among lower alkyl, lower alkoxy and halogen. Some embodiments include a third ring substituent R₃ selected from the group lower alkyl, lower alkoxy, and halogen.

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BIODEGRADABLE AZO DYES

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10 BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention concerns a method of making xenobiotic compounds more biodegradable. More specifically, it concerns biodegradable azo dyes.

15 2. General Discussion of the Background

Azo dyes are important synthetic compounds that are widely used in the dyestuff and textile industries. Unfortunately, they are not biodegradable and tend to persist in the environment unless subjected to costly physical-chemical decontamination processes. Compounds such as azo dyes which resist biodegradation are known as xenobiotics. The azo linkages or aromatic sulfo groups often found in these dyes are generally not synthesized by living organisms, which may help explain their recalcitrance to degradation. Detailed knowledge about biodegradation of these compounds in nature is limited.

Biologic waste treatment processes are sometimes more efficient and less expensive than physical-chemical waste treatment procedures, hence it would be desirable to provide a biological process using microorganisms that degrade xenobiotic azo dyes. Unfortunately, efforts to isolate such microorganisms have been largely unsuccessful in producing a commercially suitable process. Azo dye degrading *Pseudomonas* strains have been isolated from chemostat cultures by Kulla, "Aerobic bacterial degradation of azo dyes", in Microbial Degradation of Xenobiotic and Recalcitrant Compounds, Academic Press,

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Inc., London, 1981, pages 387-399 (1981). The degradation mechanism described for that *Pseudomonas* involved an oxygen-insensitive azoreductase which catalyzed the reductive cleavage of the azo group using NAD(P)H as an electron donor. Zimmerman, et al., Eur. J. Biochem., 1982, 129:197-203. Various anaerobic bacteria that degrade azo dyes have also been reported by Wuhrman, et al., Eur. J. Appl. Microbiol-Biotechnol., 1980, 9:325-338 and Meyer, "Biodegradation of synthetic organic colorants", in Microbial Degradation of Xenobiotic and Recalcitrant Compounds, supra. However, under aerobic conditions these dyes have been considered to be essentially non-biodegradable.

More recently, however, Cripps found that the fungus *Phanerochaete chrysosporium* aerobically degrades polycyclic hydrocarbons containing azo and sulfo groups. Cripps, et al., Appl. Environ. Microbiol., 1990, 56:1114-1118. That paper described several unidentified metabolites of microbially degraded Tropaeolin O, Congo Red and Orange II after incubation with crude ligninase preparations, but the possible mechanism of degradation was not explained. Other investigators have shown that *P. chrysosporium* can mineralize chloroaniline/lignin conjugates and xenobiotic molecules bound to humic acids. Haider and Martin, Soil Biol. Biochem., 1988, 20:425-429.

In spite of these advances, the degree of microbial degradation of many azo dyes has remained low. Kulla's azo dye degrading *Pseudomonas* is highly substrate specific, and requires extensive screening procedures to isolate biodegradative strains. The extreme specificity of Kulla's bacterial strains decreases their practical use in industry because industrial effluents contain mixtures of dyes. Kulla, et al., "Biodegradation of xenobiotics; experimental evolution of azo dye-degrading bacteria", in Current Perspectives in Microbial Ecology, (eds. M.J. Klug and C.A. Reddy), American Society for Microbiology, Washington, D.C., pages 663-667. Moreover, the

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Pseudomonal strains completely and irreversibly lose their biodegradative ability when grown with the specific substrate for ten generations, as disclosed at page 664 of that publication. Finally, sulfonated aromatic groups in 5 the substrate dyes disturbed the microbial degradative pathways and limited the usefulness of these microorganisms in degrading the vast quantities of industrially produced azo dyes.

Accordingly, it is an object of this invention to 10 provide azo dyes which are more completely biodegradable.

Another object of the invention is to provide such dyes which can be degraded more effectively and discarded less expensively than many previous azo dyes.

Yet another object of the invention is to provide 15 such dyes which are less harmful to the environment.

Another object of this invention is to provide azo dyes which can be degraded by microorganisms with less substrate specificity.

Another object is to provide such dyes which are 20 degraded by relatively common and genetically stable microorganisms that better retain their biodegradative capacity through successive generations.

Finally it is an object of the invention to provide an improved method of treating azo dyes in which 25 sulfonated azo compounds can be degraded.

These and other objects of the invention will be understood more clearly by reference to the following detailed description.

30

SUMMARY OF THE INVENTION

A biodegradable dye compound is disclosed which contains an azo group having a nitrogen atom linked to an aromatic ring in which the aromatic ring has a lignin-like substitution pattern that enhances biodegradability of the 35 dye compound. In preferred embodiments, the aromatic ring has a substitution pattern that resembles a syringyl or guaiacyl moiety. In especially preferred embodiments, the

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ring has a first substituent R₁ selected from the group consisting of hydroxy, lower alkoxy, or amino, particularly secondary amine. In other embodiments, the ring further includes a second substituent R₂ selected 5 from the group consisting of hydrogen, alkyl, lower alkoxy and halogen. In yet other preferred embodiments, the ring includes a third substituent R₃ selected from the group consisting of lower alkyl, lower alkoxy and halogen. In especially preferred embodiments R₁ is para to the azo 10 linkage. In other preferred embodiments R₂ is ortho to R₁. In especially preferred embodiments R₁ is para to the azo linkage and R₂ is ortho to R₁.

These and other compounds can be included in a biodegradable composition in which the azo dye of the 15 present invention is combined with an environmentally common microbe that is capable of degrading the azo dye. A wide variety of microorganisms efficiently degrade these dyes, especially microorganisms in the soil microflora. Particularly useful are a wide variety of Streptomyces species and strains of specific Streptomyces species found 20 in soil and elsewhere. Examples of two aerobic microorganisms which have been shown to degrade the dyes of the present invention are several soil Streptomyces species and Phanerochaete chrysosporium. When used in combination with Streptomyces, biodegradation is most 25 enhanced in the disclosed embodiments when R₁ is a hydroxy group para to the azo linkage, particularly if R₂ is ortho to the hydroxy.

Biodegradation with Phanerochaete is particularly 30 enhanced in some embodiments wherein R₁ is hydroxy para to the azo linkage and R₃ is absent, especially if R₂ is a group that does not have a high degree of steric hindrance. Methyl, methoxy and halogen are examples of small groups with a low steric hindrance. The presence 35 of R₃, however, can greatly enhance biodegradation in some embodiments wherein R₁ is a hydroxy group para to the azo linkage, and R₂ and R₃ are both ortho to R₁. This enhanced

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biodegradation is observed with R₃ present even in embodiments wherein R₁ is not para to the azo linkage.

In other embodiments of the invention, a pre-existing azo dye can be modified after use but before 5 disposal to render it more biodegradable by these organisms.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A shows the structure and MS spectra of the azo compound 4-(3-methoxy-4-hydroxyphenylazo)-azobenzene-10 3,4'-disulfonic acid of the present invention.

FIG. 1B shows the structure and MS spectra of the azo compound 3-methoxy-4-hydroxy-azobenzene-4'-sulfonic acid of the present invention.

FIG. 2 is a graph showing the degradation by 15 Streptomyces chromofuscus A11 of vanillic acid, sulfanilic acid, Acid Yellow 9, and two of the azo dyes synthesized in accordance with the present invention.

FIG. 3 is a graph showing the rate of degradation by Phanerochaete chrysosporium of three azo dyes and 20 sulfanilic acid.

FIG. 4A shows the absorbance spectra over time of acid yellow 9, illustrating its oxidation by P.chrysosporium ligninase.

FIG. 4B shows absorbance spectra over time of sulfanilic acid, illustrating its oxidation by 25 P.chrysosporium ligninase.

FIG. 4C shows absorbance spectra over time of azo dye 1, illustrating its oxidation by P.chrysosporium manganese peroxidase.

30 FIG. 4D shows absorbance spectra over time of azo dye 2, illustrating its oxidation by P.chrysosporium manganese peroxidase.

FIG. 5 shows the results of a high performance liquid chromatography (HPLC) of azo dye 1 before (A) and 35 after (B) incubation with P.chrysosporium manganese peroxidase, confirming degradation of the dye and aromatic compounds.

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DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Three azo dyes were tested as substrates for degradation by twelve Streptomyces species and the white rot fungus Phanerochaete chrysosporium. The three azo dyes were the commercially available acid yellow 9 (4-amino-1,1'-azobenzene-3,4'-disulfonic acid), and two synthesized dyes. The two synthesized dyes were azo dye 1 [4-(3-methoxy-4-hydroxyphenylazo)-azobenzene-3,4'-disulfonic acid] and azo dye 2 (3-methoxy-4-hydroxy-azobenzene-4'-sulfonic acid). Sulfanilic acid and vanillic acid were also tested as substrates for degradation by the twelve Streptomyces species and the white-rot fungus Phanerochaete chrysosporium. None of the Streptomyces species degraded acid yellow 9 or sulfanilic acid. The linkage of a guaiacol molecule onto acid yellow 9 or sulfanilic acid via azo-linkages resulted in dyes that were decolorized by five of the twelve Streptomyces strains. These Streptomyces were those that could also attack vanillic acid, which has the same ring substitution pattern (4-hydroxy-3-methoxy) as guaiacol. While P.chrysosporium transformed both acid yellow 9 and sulfanilic acid, the two guaiacol-substituted azo dyes were decolorized more readily by P.chrysosporium than the corresponding unsubstituted molecules. Ligninase and manganese peroxidase preparations from the P.chrysosporium culture were apparently involved in the degradation.

Source of Materials

Sulfanilic acid, guaiacol, sodium nitrite and 4-hydroxy-3-methoxybenzoic acid (vanillic acid) were purchased from the Aldrich Chemical Co. The azo dye 4-amino-1,1'-azobenzene-3,4'-disulfonic acid (acid yellow 9) was purchased from Sigma Chemical Co. Two additional azo dyes were synthesized by attaching guaiacol through an azo linkage to acid yellow 9, forming 4-(3-methoxy-4-hydroxyphenylazo)-azobenzene-3,4'-disulfonic acid (azo dye

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1), or to sulfanilic acid, forming 3-methoxy-4-hydroxy-azobenzene-4'-sulfonic acid (azo dye 2). Structures and MS fragmentations of the dyes are shown in Figs. 1a and 1b. Purity of both synthesized dyes was determined by TLC 5 and HPLC analysis; no significant impurities were detected. The HPLC integration data showed the purity was approximately 98% for azo dye 1 and 97% for azo dye 2.

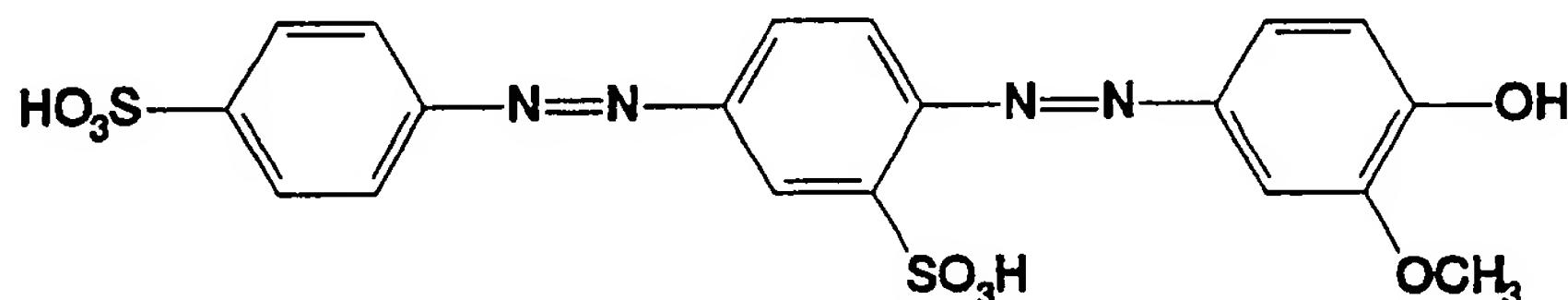
Synthesis of Azo Dye 1

4-(3-methoxy-4-hydroxyphenylazo)-azobenzene-3,4'-disulfonic acid

Azo dye 1 was synthesized by dissolving 4-Amino-azobenzene-3,4'-disulfonic acid sodium salt (0.76 g) in 5% sodium hydroxide (8 ml), and a solution of sodium nitrite 15 (0.14 g in 0.5 ml of water) was added. Crushed ice (10 g) and concentrated HCl (1.8 ml) was introduced to the solution, which was then vigorously stirred for 15 minutes. To the cooled guaiacol solution (0.25 g dissolved in 3.2 ml 5% sodium hydroxide) the diazotised 20 yellow 9 solution was added portionwise over 15 minutes with mechanical stirring. Saturated sodium chloride solution was added (15 ml), and the mixture was left to crystallize overnight at 5°C. The crystalline product was filtered, washed with acetone and ether, and dried in air. 25 Dark brown crystals (0.98 g) were collected. (86.6% of theoretical yield).

The chemical structure of azo dye 1, 4-(3-methoxy-4-hydroxyphenylazo)-azobenzene-3,4'-disulfonic acid, is:

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as supported by the MS spectra shown in Fig. 1A.

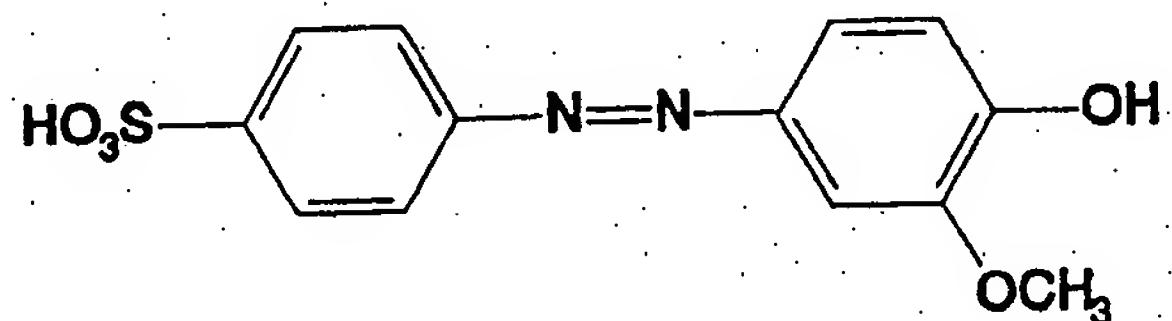
Synthesis of Azo Dye 2

3-methoxy-4-hydroxy-azobenzene-4'-sulfonic acid

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Azo dye 2 was synthesized by suspending sulfanilic acid (1.73 g) in 23 ml of water, and 8 ml of 5% NaOH were added. The mixture was stirred until the acid dissolved and then sodium nitrite solution (0.7 g in 2 ml H₂O) was added. The solution was poured on a crushed ice (25 g) and concentrated HCl (2 ml) mixture and mixed until copious precipitation took place (KJ starch test was positive). The diazotized sulfanilic acid was added portionwise to the cooled guaiacol solution (1.24 g in 20 ml 5% sodium hydroxide) with stirring. NaCl (20 g) was added and stirring was continued for 30 minutes at room temperature. The crystalline deep orange precipitate was filtered off and washed with ethanol and ether; 2.62 g of the product was obtained (64.4% of theoretical yield).

The chemical structure of azo dye 2, 3-methoxy-4-hydroxy-azobenzene-4'-sulfonic acid, is:



as supported by the MS spectra shown in Fig. 1B.

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Azo Dyes 3-19

Analogous methods of synthesis were used to prepare azo dyes 3-19. The structures of these compounds are shown below.

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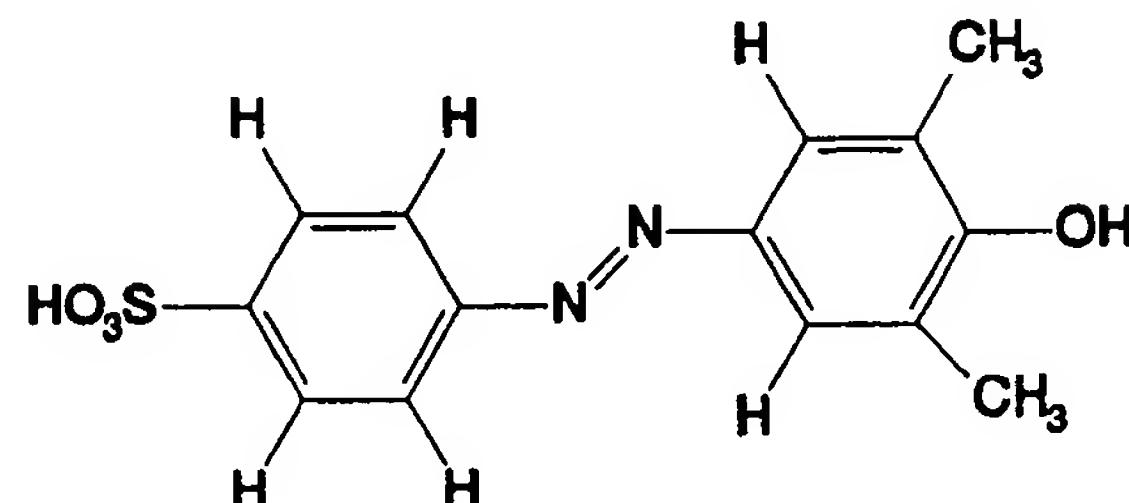
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- 8A -

Azo Dye 3

3,5-dimethyl-4-hydroxy-azobenzene-4'-sulfonic acid

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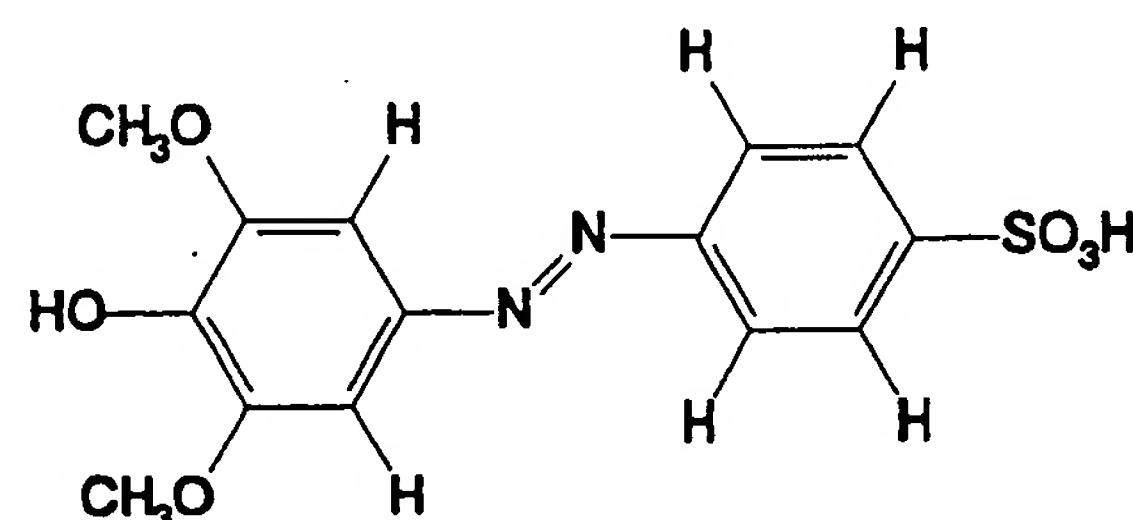


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Azo Dye 4

3,5-dimethoxy-4-hydroxy-azobenzene-4'-sulfonic acid

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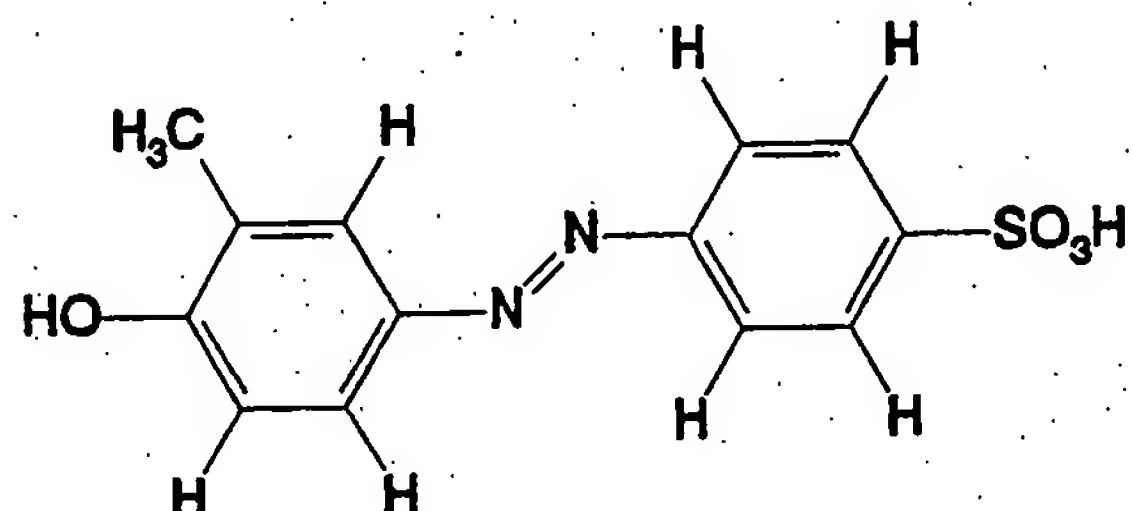
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Azo Dye 5

3-methyl-4-hydroxy-azobenzene-4'-sulfonic acid

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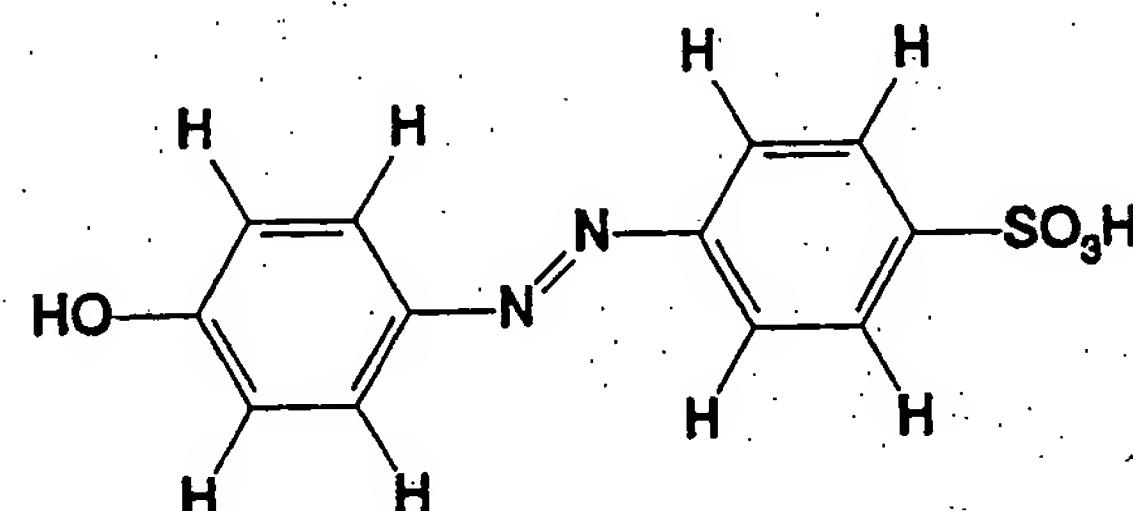


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Azo Dye 6

4-hydroxy-azobenzene-4'-sulfonic acid

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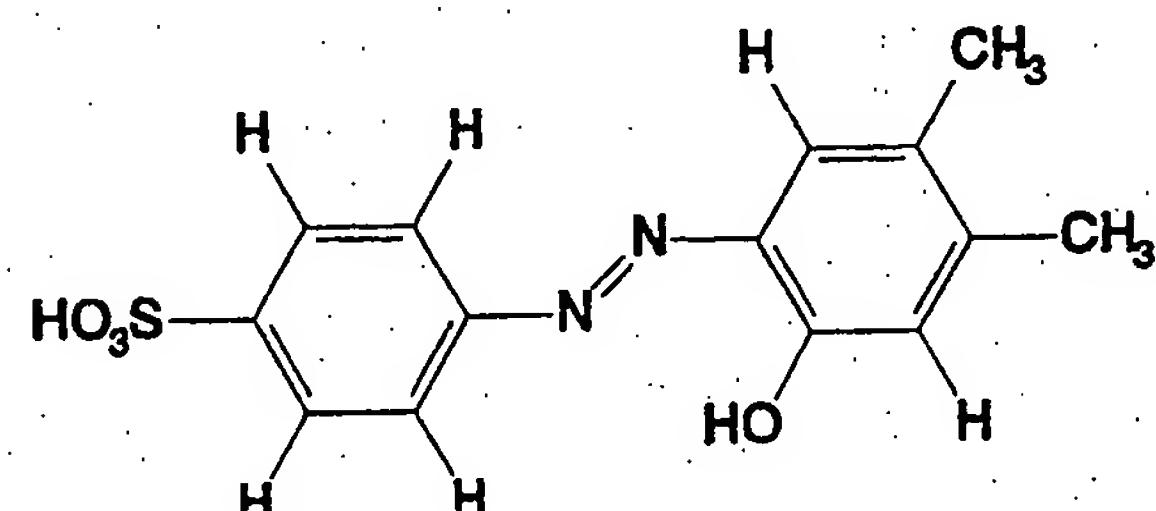


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Azo Dye 7

2-hydroxy-4,5-dimethyl-azobenzene-4'-sulfonic acid

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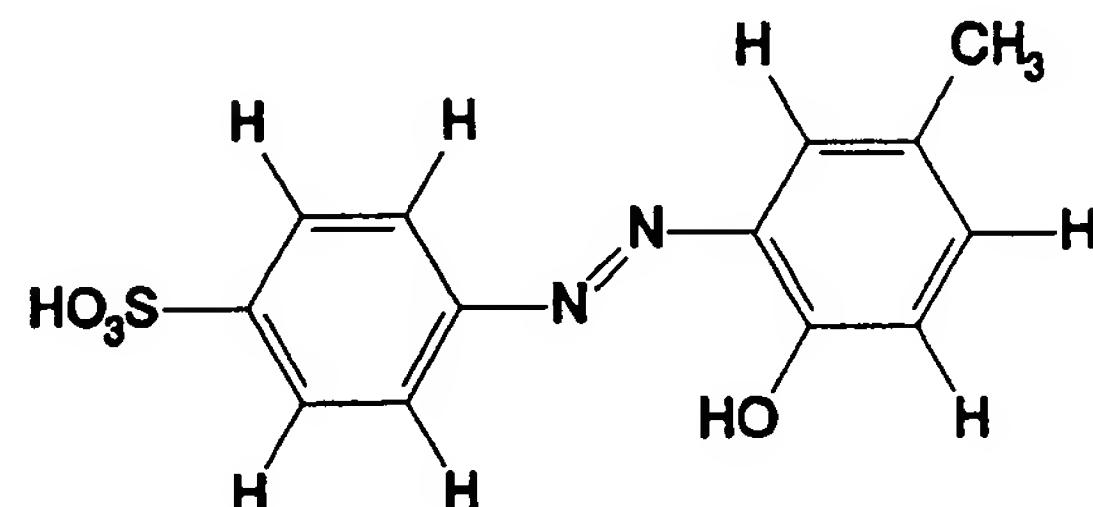


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Azo Dye 8
2-hydroxy-5-methyl-azobenzene-4'-sulfonic acid

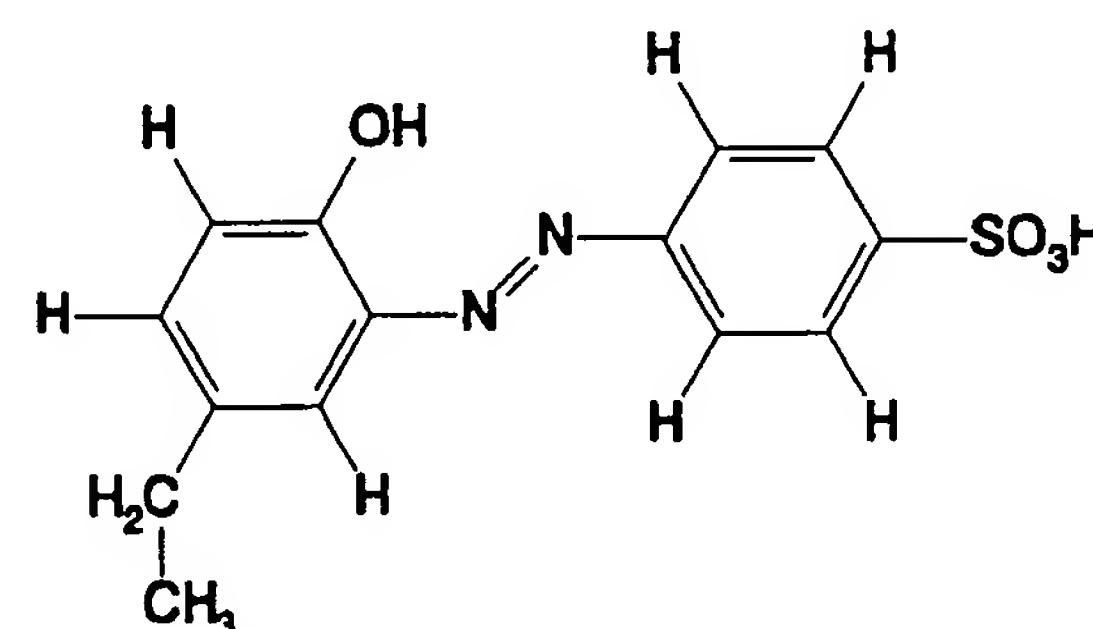
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Azo Dye 9
2-hydroxy-5-ethyl-azobenzene-4'-sulfonic acid

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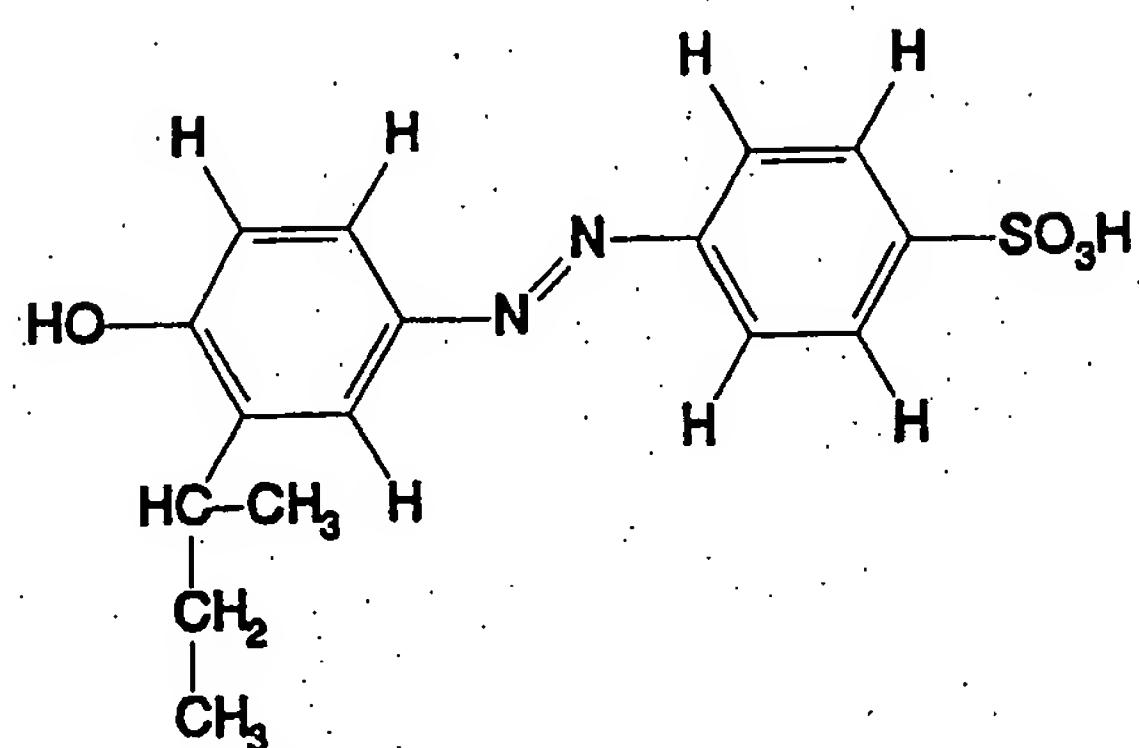
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Azo Dye 10

3-sec-butyl-4-hydroxy-azobenzene-4'-sulfonic acid

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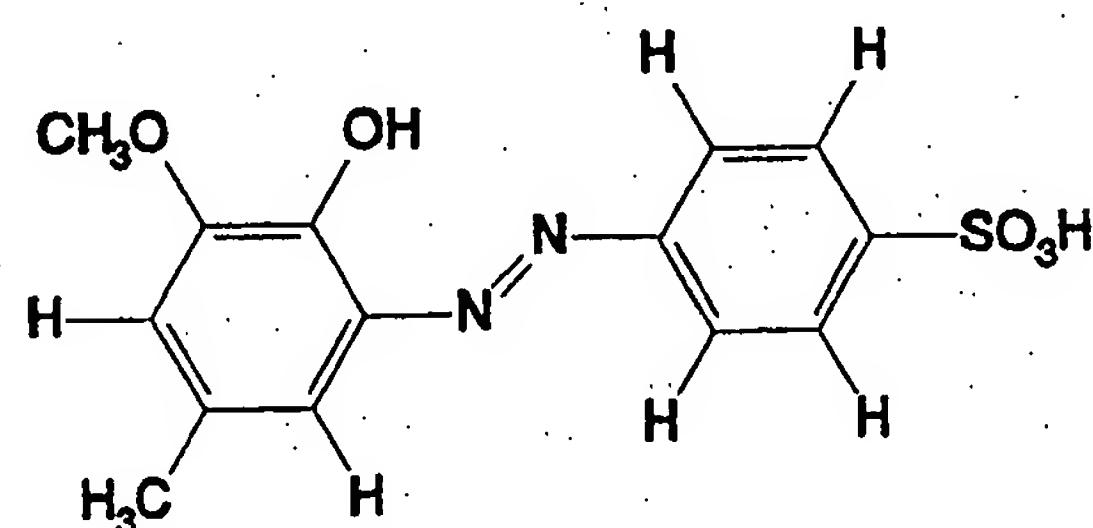
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Azo Dye 11

2-hydroxy-3-methoxy-5-methyl-azobenzene-4'-sulfonic acid

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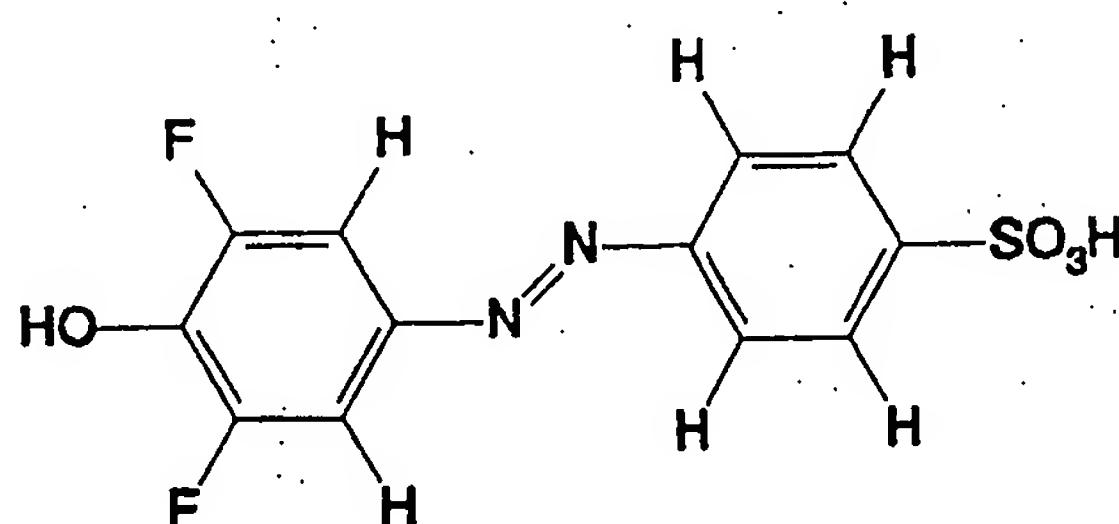


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Azo Dye 12

3,5-difluoro-4-hydroxy-azobenzene-4'-sulfonic acid

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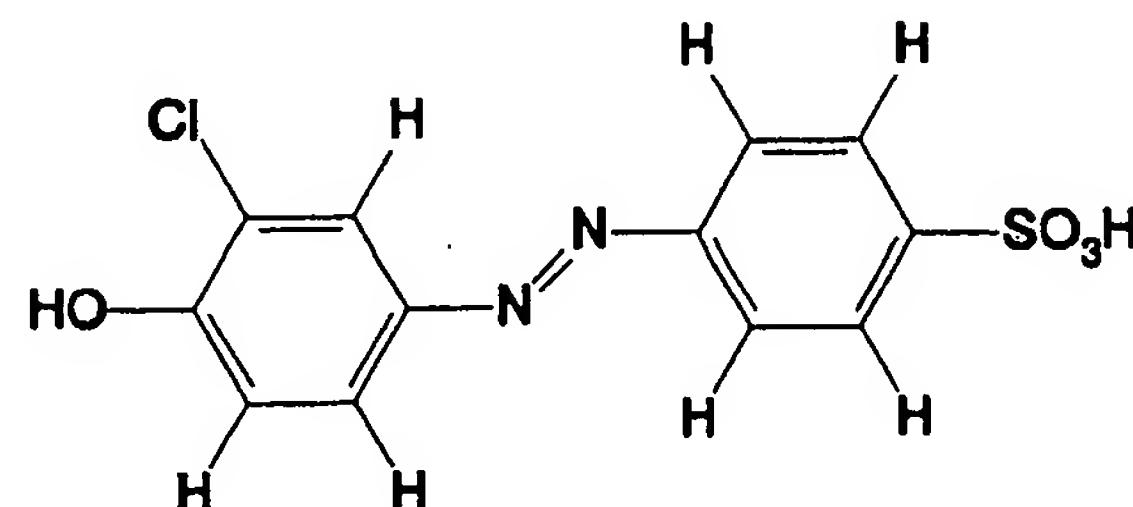


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Azo Dye 13
3-chloro-4-hydroxy-azobenzene-4'-sulfonic acid

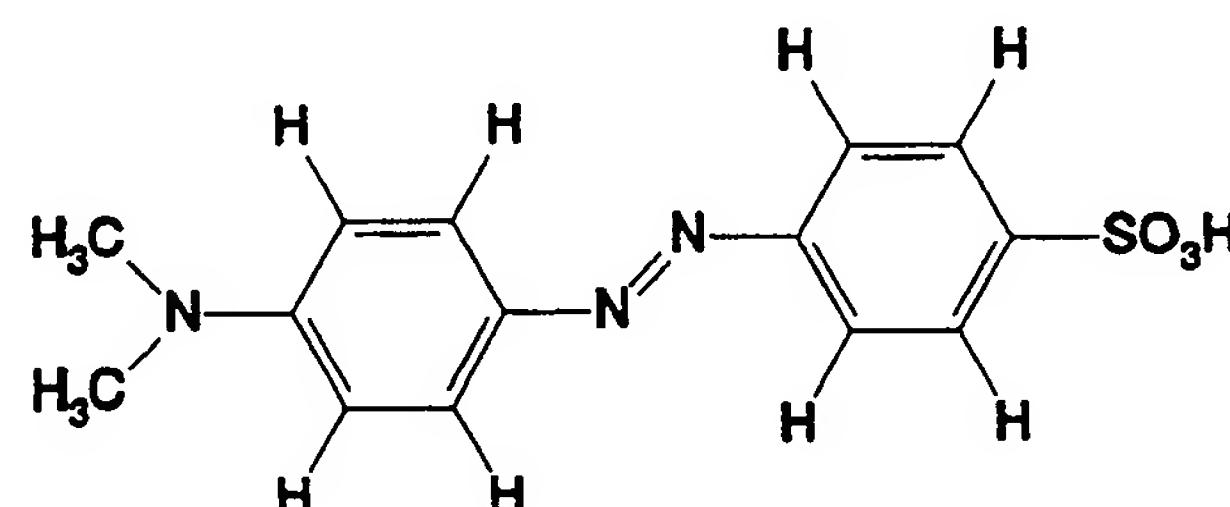
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Azo Dye 14
4-dimethylamino-azobenzene-4'-sulfonic acid

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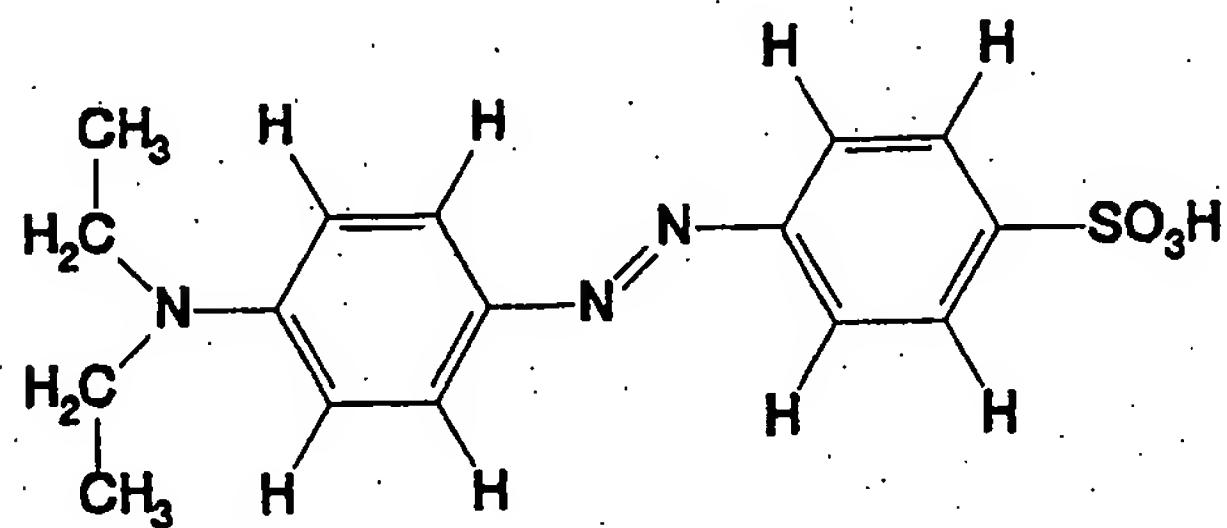


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Azo Dye 15
4-diethylamino-azobenzene-4'-sulfonic acid

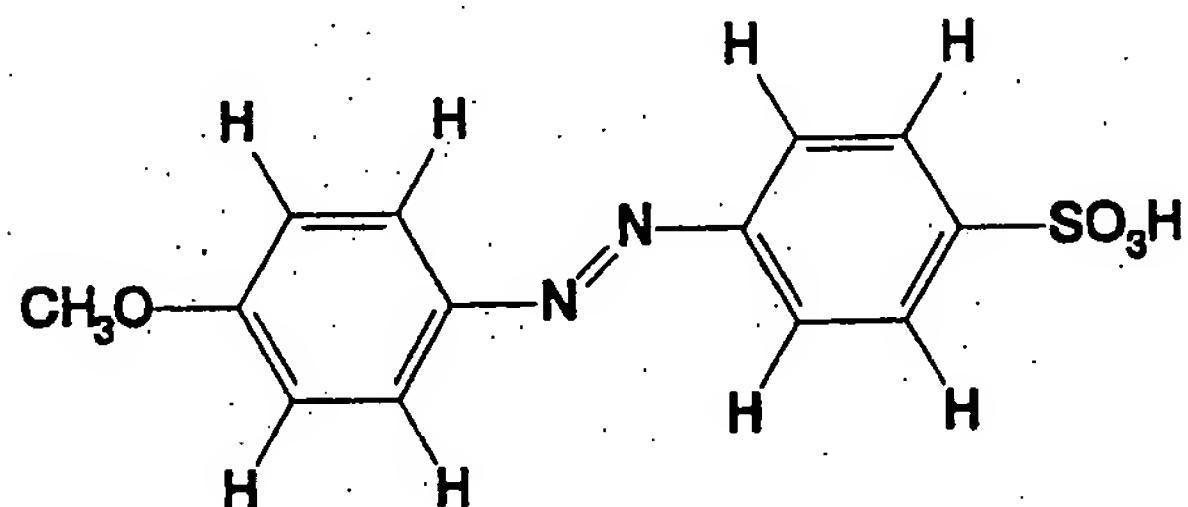
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Azo Dye 16
4-methoxy-azobenzene-4'-sulfonic acid

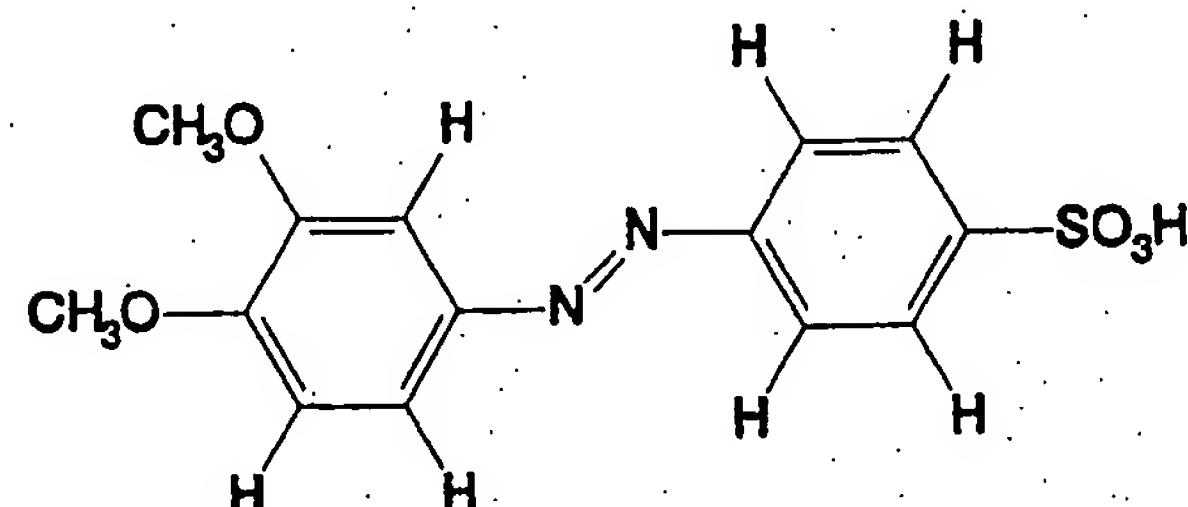
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Azo Dye 17
3,4-dimethoxy-azobenzene-4'-sulfonic acid

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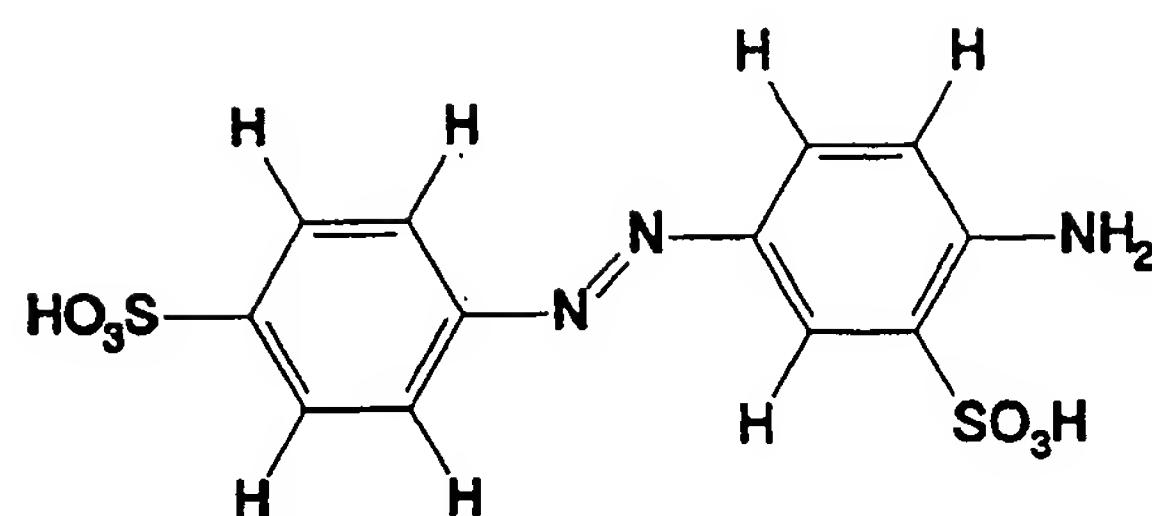


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Azo Dye 18

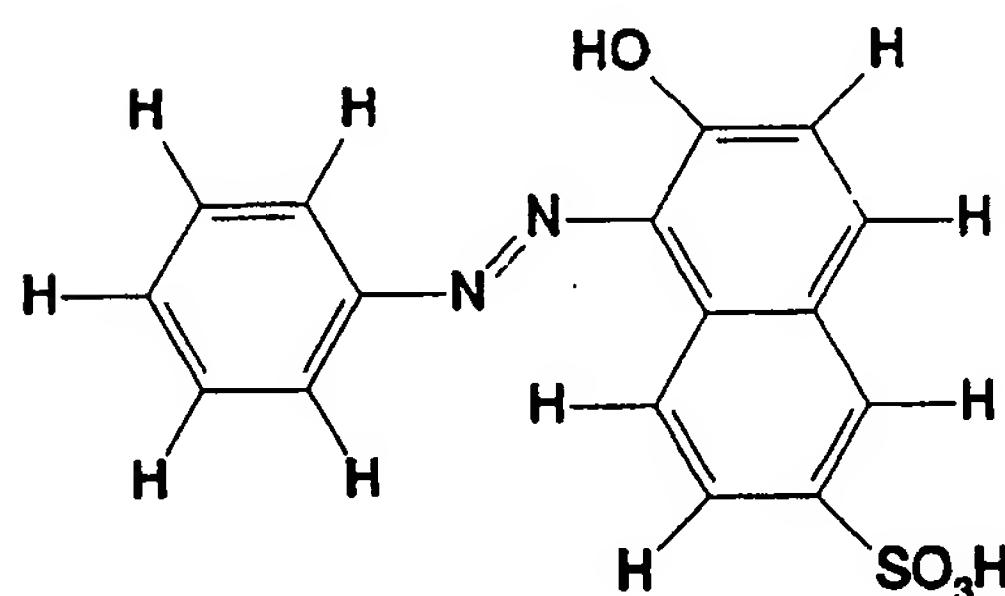
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Azo Dye 19

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Microorganisms and Culture Maintenance

Twelve wild-type actinomycetes were selected from 20 strains isolated from higher termites in Kenya. Pasti and Belli, FEMS Microbiol. Lett., 1985, 26:107-112. All 5 strains have been identified as *Streptomyces*, based on the key of Williams, et al. J. Gen. Microbiol., 1983, 129:1815-1830. *Streptomyces viridosporus* T7A (ATCC 39115) was isolated from soil by D.L. Sinden (M.S. thesis, University of Idaho, Moscow, 1979). *Streptomyces badius* 10 252 (ATCC 39117) was isolated from soil by Phelan et al. (Can. J. Microbial., 1979, 27:636-368) and *Streptomyces* SR-10 is a protoplast fusion recombinant derived from a cross between *S.viridosporus* T7A and *S.setonii* 75Vi2. Pette and Crawford, Appl. Environ. Microbiol., 1984, 47:439-440. 15 Stock cultures of the Kenyan isolates were maintained at 4°C, after growth and sporulation at 37°C on the following medium in grams per liter of deionized water: NH₄NO₃, 1; KH₂PO₄, 0.4; yeast nitrogen base (Difco), 0.67; yeast extract (Difco), 0.2; lactose, 15; bacto-agar (Difco), 18. 20 *S.viridosporus* T7A, *S.badius* 252 and *S.* SR-10 were maintained at 4°C, after growth and sporulation at 37°C on yeast extract-malt extract dextrose agar, as in Pridham and Gottlieb, J. Bacteriol., 1948, 56:107-114. Stock cultures were subcultured every 2 to 10 weeks, and 25 distilled water suspensions of sporulated growth were used as initial inocula in all experiments.

Phanerochaete chrysosporium BKM-F-1767 (ATCC 24725) was obtained from The Forest Products Laboratory, Madison, WI. The fungus was maintained and spore inocula 30 were prepared as previously described by Huynh and Crawford, FFMS Microbiol. Lett., 1985, 28:119-123.

All publications describing isolation of the *Streptomyces* and *Phanerochaete*, and all publications 35 describing culture maintenance, are incorporated by reference.

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Culture Conditions

Each *Streptomyces* species was grown in a cotton-plugged 250 ml flask containing 25 ml of the following medium: 0.2 M Tris buffer (pH 7.6), 100 ml; vitamin-free Casamino acids (Difco), 1.0 g; thiamine, 100 µg; biotin, 100 µg; D-glucose, 2 g; deionized water, 900 ml. Thiamine, biotin and D-glucose were filter-sterilized and added to the autoclaved medium, as in McCarthy and Broda, *J. Gen. Microbiol.*, 1984, 130:2905-2913. The dyes were filter-sterilized and added at 0.005% (w/v) to the autoclaved basal medium. Three replicates of every culture were incubated, and each strain was grown in media supplemented individually with every substrate. Replicate sterile controls also were run in each experiment. Cultures were incubated at 37°C for 14 days with shaking (200 rpm). Three replicates for each strain growth were incubated in only the basal medium as well.

P.chrysosporium was grown in a cotton-plugged 500 ml flask containing 250 ml defined medium (Jeffries, et al., *Appl. Environ. Microbiol.*, 1981, 42:290-296), with the addition of 75 mg adenine (6-aminopurine) and 27 mg L-phenylalanine per liter. This addition accelerated the growth of the fungus without inhibiting ligninase activity. Four substrates were tested: sulfanilic acid, acid yellow 9 and the two synthesized azo dyes; each was separately added at a concentration of about 0.02% (w/v). Cultures were incubated at 37°C for 7 to 15 days with shaking (250 rpm). Solid agar media were also employed. The medium was 3.0% (w/v) malt extract (Difco Laboratories) agar dispensed in petri plates. The medium also contained 120 mg per liter of specific azo dye.

Spectrophotometric Assay

A one ml sample of actinomycete culture medium was centrifuged and then diluted 2.5-fold with water, or 1.0 ml of fungal supernatant was centrifuged and diluted 5-fold with 10 mM sodium 2,2-dimethylsuccinate buffer

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(DMS) of pH 4.5. Azo dye substrate present was then measured spectrophotometrically with a Hewlett-Packard 8452 diode array spectrophotometer operated by a PC Vectra computer equipped with HP's MS™-DOS/UV-VIS software. To be certain that changes in substrate spectra were not due to pH variations, the effects of pH on the visible absorption of each compound were also assayed within physiological pH range in the culture media. While the spectra of sulfanilic acid (Max abs at 250nm), vanillic acid (Max abs at 252nm and 286nm) and acid yellow 9 (Max abs at 336nm) were unaffected by pH over the tested pH range, the spectra of the two novel azo dyes were changed as evidence by shifts of their Abs_{max} . Thus, the spectrophotometric assays for these dyes were carried out at their isobestic points. These were, respectively, 450nm for azo dye 1 and 400nm for azo dye 2.

High Performance Liquid Chromatography Analysis

Degradation of the dyes and aromatic compounds was confirmed by high performance liquid chromatography. A Hewlett-Packard HP 1090 Liquid Chromatograph equipped with a HP 40 diode array UV-VIS detector and automatic injector was used. The chromatograph was controlled by an HP 9000 series 300 computer which used HP 7995 A ChemStation software. A reverse phase column from Phenomenex (Rancho Palos Verdes CA., type Spherex 5 C 18 size 250 x 2.0 mm, serial number PP/6474A) was used. Each 15 minute analysis used a solvent gradient of acetonitrile (solvent A) and 10 mM DMS buffer pH 4.5 (solvent B), with the following conditions: 0 to 5 minutes 100% A; 5 to 12 minutes 25% A 75% B; 12 to 15 minutes 100% B; post time 2 minutes injection volume 10 μ l. Absorption was measured at 250, 325, 350, 400 and 450 nm, and spectra were collected automatically by the peak controller.

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Streptomyces species peroxidases were prepared and assayed using 2,4-dichlorophenol (2,4-DCP) from Sigma Chemical Company as a substrate, as described in Ramachandra, et al., Appl. Environ. Microbiol., 1988, 54:3057-3063. *P.chrysosporium* BKM-F-1767 was grown in a 20-liter carboy containing one liter of nitrogen-limited defined medium (BII-medium), as described by Paszczynski, et al., Arch Biochem. Biophys., 1986, 44:750-765. Preparation and assay of ligninase and manganese peroxidase from these *P.chrysosporium* cultures were carried out as previously reported by Paszczynski, et al., Methods Enzymol., 1988, 161:264-270.

Oxidation by Enzyme Preparations

15 Extracellular enzyme preparations of the *Streptomyces* species were not observed to produce any detectable decolorization of substrates.

20 The decolorization of acid yellow 9 azo dye and the oxidation of sulfanilic acid by extracellular preparations of *Phanerochaete chrysosporium* enzymes is shown in Figs. 4A and 4B, respectively. Fig. 4A shows oxidation of acid yellow 9 by ligninase. The reaction conditions were 0.2mM hydrogen peroxide, 50mM sodium tartrate buffer, pH3, 10 μ g of dye, and 0.6 units of enzyme 25 (20 μ l) in total volume of 1 ml. Cycle time was 30 seconds with the last measure after 15 minutes. During a period of 15 minutes the ligninase exhibited a stable activity which decolorized about 3 micrograms out of 10 micrograms of acid yellow 9 in the reaction mixture.

30 Fig. 4B shows oxidation of sulfanilic acid which was transformed slowly by ligninase, with an increase in absorbance at about 480nm. The reaction conditions were the same as for acid yellow 9. No decolorization of the synthesized dyes by ligninase was detected.

35 Figs. 4C and 4D show decolorization of azo dyes 1 and 2 by the manganese peroxidase of *P.chrysosporium*. Reaction conditions were 0.2 mM hydrogen peroxide, 50 mM

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sodium tartrate buffer, pH5, 10 μ g dye, 1 unit of enzyme, 1 mM MnSO₄ in a total volume of 1 ml. Cycle time was 10 seconds, with the last measure after three minutes of incubation. Oxidation of azo dye 1 by manganese peroxidase resulted in new peak formation at 355nm. After one hour incubation of azo dye 1 with manganese peroxidase the dye was almost completely degraded comparable to a control chromatogram (Fig. 5). No decolorization of acid yellow 9 or oxidation of sulfanilic acid by manganese peroxidase was detected.

Biotransformation of Microorganisms

Table 1 shows the substrate utilization pattern of the *Streptomyces* species after a growth period of 14 days. Only six strains (A4, A10, A11, A12, A13, and A14) significantly degraded vanilllic acid, while none degraded sulfanilic acid or acid yellow 9 to a detectable extent. Significant degradation was considered degradation greater than about 10%. This result confirms that the compounds characterized by aromatic sulfo group and azo linkages are quite recalcitrant. However, 5 strains (A10, A11, A12, A13, and A14) significantly degraded both the two new azo dyes. Moreover, azo dye 2 was degraded by these strains to a larger extent than azo dye 1.

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TABLE 1

Percent substrate removed by cultures of *Streptomyces* and *Phanerochaete* during a growth period of 14 and 7 days respectively

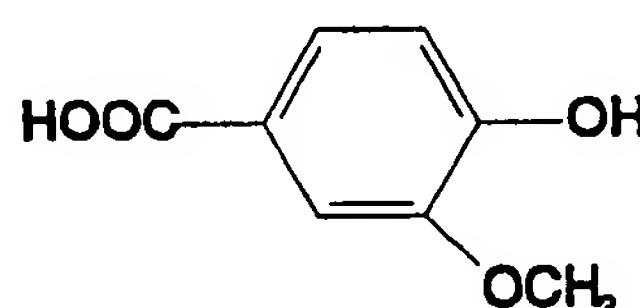
	Strain	Sulfanilic acid	Vanilllic acid	Yellow Azo dye #9	Azo dye #1	Azo dye #2
35	S. chromofuscus A2	-	-	-	-	-
	S. diastaticus A3	-	-	-	-	-
	S. rochei A4	-	100	-	-	-
40	S. chromofuscus A6	-	-	-	-	-
	S. cyaneus A7	-	-	-	-	-
	S. chromofuscus A8	-	-	-	-	-
	S. rochei A10	-	91	-	51	74

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	S. chromofuscus A11	-	100	-	56	89
	S. diastaticus A12	-	58	-	27	30
	S. diastaticus A13	-	34	-	15	21
	S. rochei A14	-	72	-	43	72
5	S. chromofuscus A20	-	5	-	1	11
	S. viridosporus T7A	-	3	-	1	9
	S. SR-10	-	-	-	-	-
	S. badius 252	-	7	-	9	18
10	P. chrysosporium	68	n.d.	79	93	94

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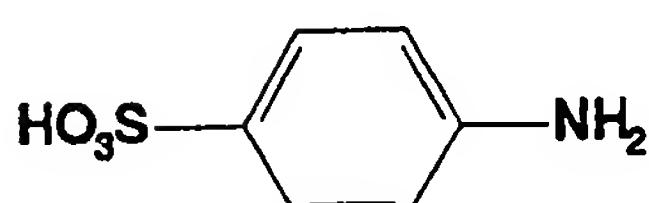
Figure 2 shows the pattern of degradation of each compound by strain *S.chromofuscus* A11 versus time, as a typical example. The graph shows the degradation of vanillic acid (□), sulfanilic acid (▽), acid yellow 9 (○), and azo dyes 1 (●) and 2 (▼). The medium contained 0.2 M Tris buffer (pH 7.6), 100 ml; vitamin-free casamino acids, 1.0 g; thiamine, 100 µg; biotin, 100 µg; D-glucose, 2 g; and deionized water, 900 ml. Starting substrate concentrations were 50 ppm. *S.chrysosporium* degraded sulfanilic acid and acid yellow 9, but only to a limited extent. The vanillic acid, in contrast, was rapidly and thoroughly degraded, as were azo dyes 1 and 2. The ring substitution patterns for vanillic acid, sulfanilic acid and guaiacol are shown below:



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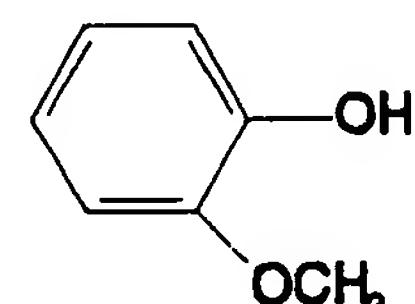
Vanillic acid
4-hydroxy-3-methoxy benzoic acid

35



40

Sulfanilic acid



Guaiacol

- 17A -

It appears that the linkage of a guaiacol moiety into azo dye yellow 9 allowed Streptomyces species capable of utilizing vanillic acid to decolorize an azo dye that 5 the Streptomyces could not otherwise transform. The only vanillic acid degrader that could not attack either azo dyes 1 or 2 was S.rochei A4, possibly because this strain catabolizes vanillic acid by attacking its carboxylic acid group, a substituent absent in the guaiacol moiety.

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Hence, utilization of the two dyes appears to start at the guaiacol substituent, but the pathway used by these Streptomyces remains to be elucidated.

The Phanerochaete fungus almost completely (90%)
5 degraded azo dyes 1 and 2 after a growth period of seven days as shown in Figure 3. The graph shows decolorization or removal by P.chrysosporium of sulfanilic acid (▽), acid yellow 9 (○), azo dye 1 (●), and azo dye 2 (▼). Agitated cultures were grown at 37°C in a mineral medium
10 supplemented with phenylalanine, and starting dye concentrations were 150-200 ppm. There was a characteristic lag of 80-90 hours prior to degradation of any of the compounds by the fungus due to slower growth compared to the Streptomyces. On the solid medium,
15 P.chrysosporium behaved similarly leaving some undegraded color after two weeks of growth. P.chrysosporium apparently degrades using its ligninolytic enzymes.

In this work several times higher concentrations of azo dyes were used than reported earlier by Cripps.
20 The ability of this organism to oxidize sulfonated azo aromatic compounds was also tested, shown in Fig. 3 and Table 1. The maximum rate of decolorization occurred on the fourth day of growth in the BII medium for all of the compounds. However, in the cultures with yellow 9 or
25 sulfanilic acid, as assayed by spectrophotometric and HPLC analysis, some undegraded dye remained in the medium after decolorization ended. Yet using HPLC, the inventors were not able to detect any residual substrate in the culture broth after 1 week of growth, even though color was still
30 present in the culture filtrates. One explanation is the finding of Kulla, et al., supra, who found that in cultures of Pseudomonas which were actively degrading azo dyes, secondary oxidative coupling occurred between sulfonated and nonsulfonated phenols, giving dead-end
35 polymers resistant to further degradation.

In testing which, if either, of the ligninolytic peroxidases of P.chrysosporium was involved in the

- 19 -

degradation of these azo compounds, it was found that ligninase oxidized yellow 9 and sulfanilic acid (Fig. 4A and B), while manganese peroxidase oxidized azo dyes 1 and 2 (Fig. 4C and D). The HPLC analysis of the reaction mixture after incubation of azo dye 1 with manganese peroxidase revealed polymorphic reaction products (Fig. 5B). Oxidation of sulfanilic acid by ligninase produced a purple unstable product, which upon exposure to air precipitated. During a 15-minute incubation period, oxidation of yellow 9 or sulfanilic acid by manganese peroxidase was not detected, nor the oxidation of azo dye 1 or 2 by ligninase. Thus, it is possible that ligninases may cooperate in the degradation of azo dyes 1 and 2.

Azo dyes 1 and 2 were decolorized to a greater extent by P.chrysosporium than was acid yellow 9 (Table 1). Even greater decolorization was noted during the growth of S.chromofuscus A11 (Fig. 2). These results show that linkage of a guaiacol molecule into the dye structure increased its susceptibility to degradation. Azo dye structures are typically conjugated multi-unsaturated systems. This makes it possible to change only one fragment of the molecule and yet have the entire conjugated system become accessible to enzymatic attack, particularly with microorganisms like white-rot fungi that use oxidative enzymes that generate cation radicals. This finding has general application for synthesizing more easily biodegradable azo dyes and other recalcitrant compounds in accordance with the present invention.

30

Biotransformation of Azo Dyes 3-19

Table 2 shows the concentrations of azo dyes 2-19 after cultivation with P. chrysosporium for ten days on mineral medium.

- 20 -

TABLE 2
Concentration of Substrates After Ten Days Cultivation
with P.chrysosporium

		Beginning Concentrations			
	Azo-comp number	100 ppm	150 ppm	200 ppm	300 ppm
10	2	2.493	6.350	10.068	7.318
	3	0.358	1.223	1.894	114.772
	4	1.637	3.763	4.218	16.323
	5	6.084	9.870	14.500	22.680
15	6	12.909	14.003	33.309	39.326
	7	62.893	41.044	10.200	246.044
	8	16.079	28.401	29.704	135.680
	9	81.911	109.822	32.044	153.733
	10	8.135	15.279	46.588	42.897
20	11	6.795	14.416	30.075	21.765
	12	2.368	44.227	6.160	198.240
	13	7.734	20.776	28.216	20.096
	14	4.411	11.843	15.529	36.800
	15	1.901	10.589	17.931	17.791
25	16	67.801	92.683	179.300	219.079
	17	29.803	58.942	93.250	136.057
	18	8.387	20.342	40.875	77.099
	19		2.938		252.592

30 Table 3 shows the concentrations of azo dye substrates after cultivation with Streptomyces strains A10, A11, A12, and A13.

35 TABLE 3
Percent Degradation of Substrates After
Streptomyces Cultivation

	Substrates	Wavelengths	Degradation (%)			
			A10	A11	A12	A13
45	2	396	77	73	43	18
	3	396	68	73	39	10
	4	416	79	83	39	8
	5	386	9	20	3	8
	6	376	-	-	-	-
50	7	430	-	-	-	-
	8	422	-	-	-	-
	9	420	-	-	-	-

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	10	394	11	16	7	6
	11	420	4	9	-	-
	12	376	-	-	-	-
	13	376	2	5	1	1
5	14	466	-	-	-	-
	15	474	2	3	1	1
	16	350	-	-	-	-
	17	408	-	-	-	-
	18	386	-	-	-	-

10

- 21 -

- = no degradation

The Streptomyces were grown on the same media and under the same conditions as previously described.

5 Spectroscopic analysis of substrates 14, 15, 16, 18 was unaffected by pH over the tested pH range. However, the Abs_{max} of the substrates 1 to 12 shifts with pH changes. Thus the spectrophotometric assays for substrates 1 to 12 were carried out at their specific isosbastic points.

10 Degradation was calculated as percent of substrates removed from culture broth, considering the evaporation factor (around 10%). The substrates' concentrations have been calculated versus standard curves prepared for each dye (0-50 μg) at the chosen wavelength; 15 standard curves over the tested concentrations were linears.

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Lignin-Like Structures

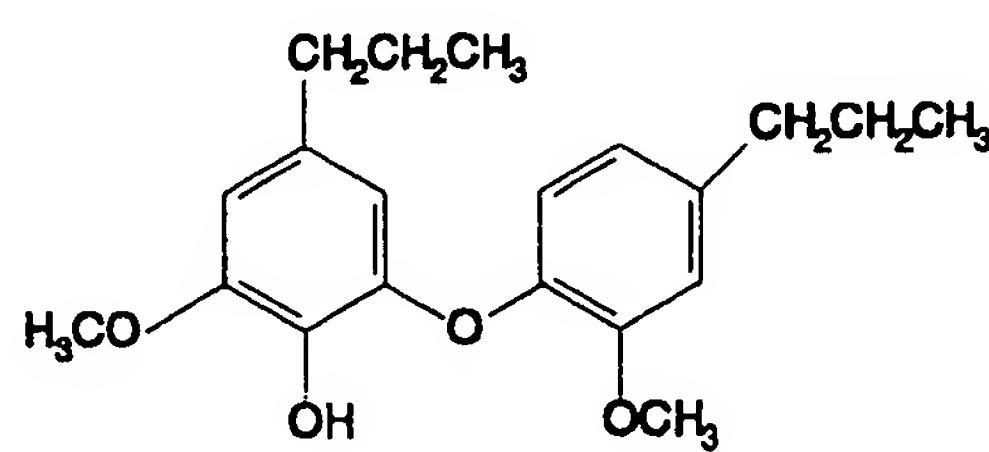
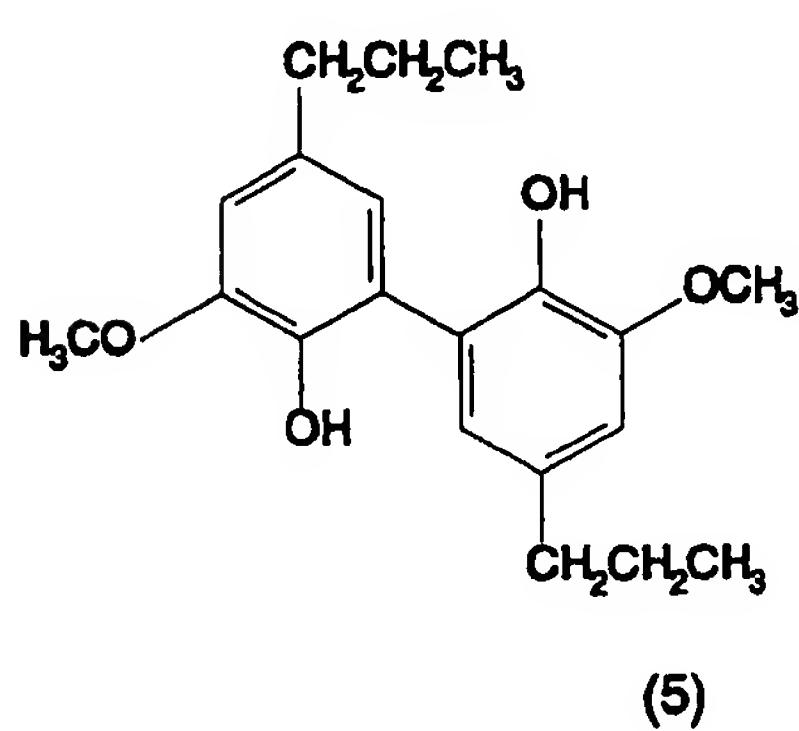
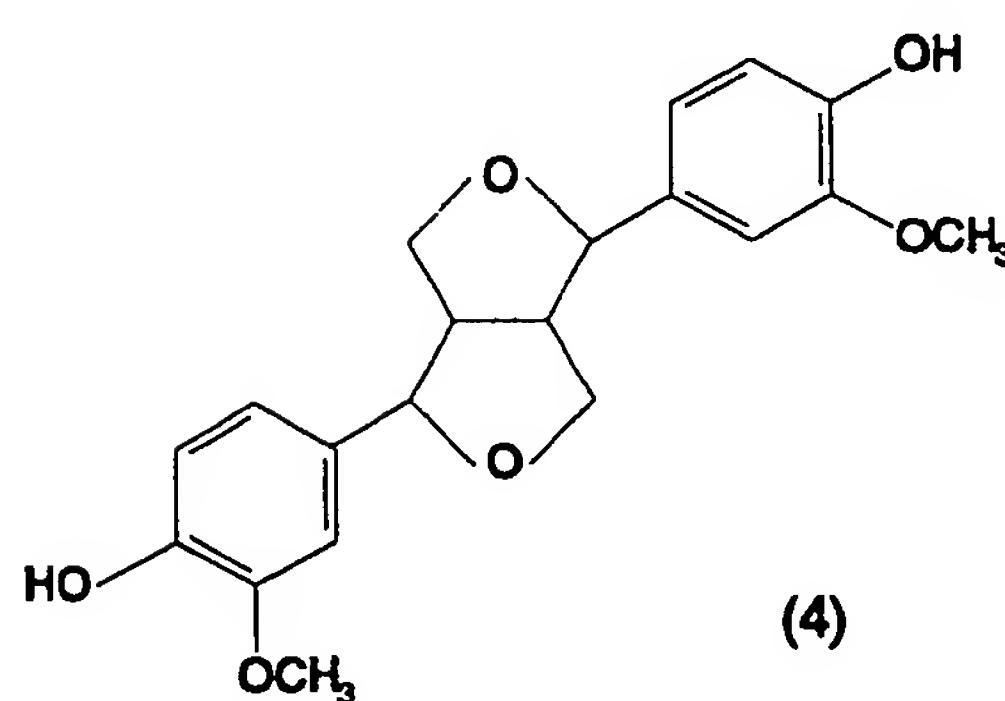
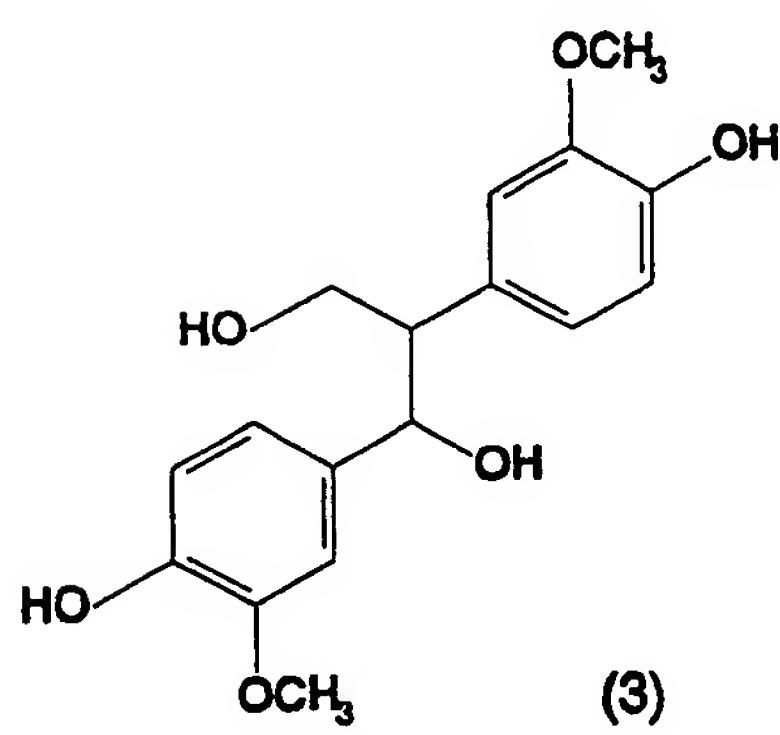
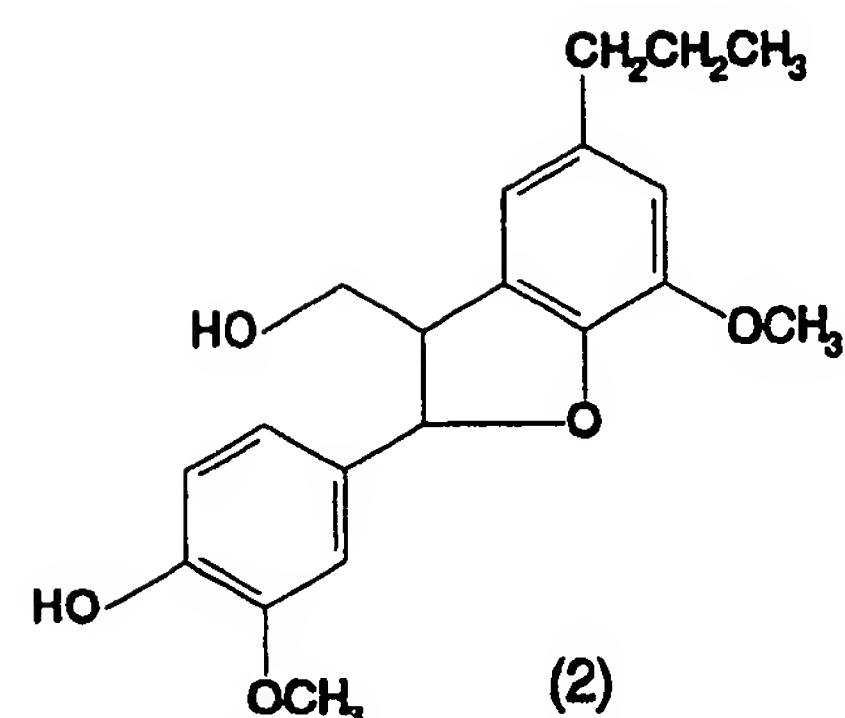
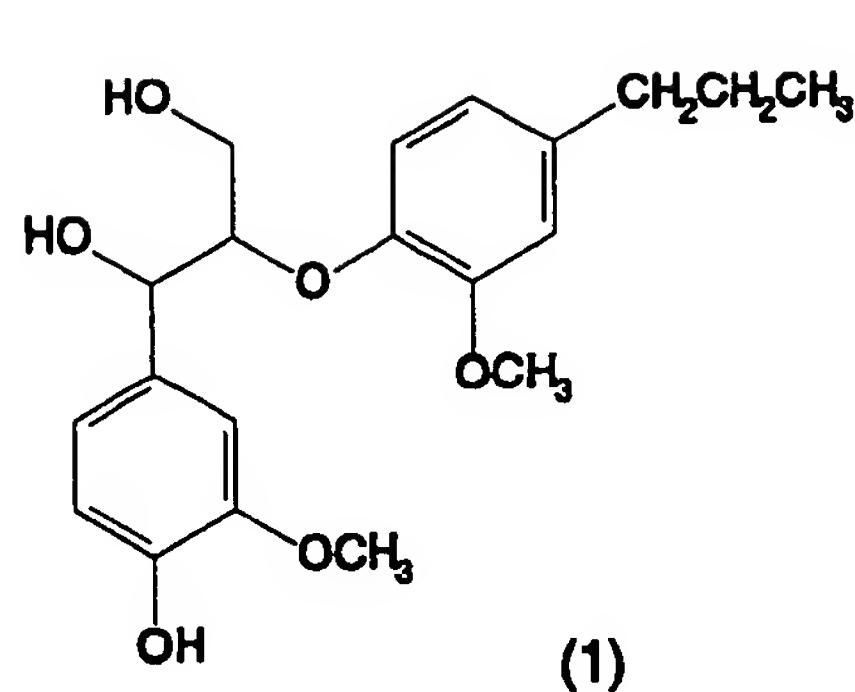
As the data in Tables 1-3 demonstrate, the biodegradability of xenobiotics, such as azo dyes, can be enhanced by attaching lignin-like structures to them.

5 Lignin-like structures are those that are contained in lignin and which enhance biodegradability of xenobiotic azo dyes when they are attached to them. Lignin-like structures also include analogous chemical structures

10 which are not known to be in lignin, yet sufficiently resemble lignin structures to provide enhanced biodegradability.

Chemical and spectrometric studies of softwood lignin indicate that lignin is an aromatic polymer in which the monomeric guaiacylpropane units are connected by both ether and carbon-carbon linkages. Several substructures in lignin macro-molecules include guaiacylglycerol- β -aryl ether (β -O-4' substructure 1) which is the most abundant interphenylpropane linkage (40-60%) in lignin, followed by phenylcoumaran (β -5' substructure 2; 10%), diarylpropane (β -1' substructure 3; 5-10%), pinoresinol (β - β ' substructure 4; 5%), biphenyl (5-5' substructure 5; 10%), diphenyl ether (4-O-5' substructure 6; 5%), and others.

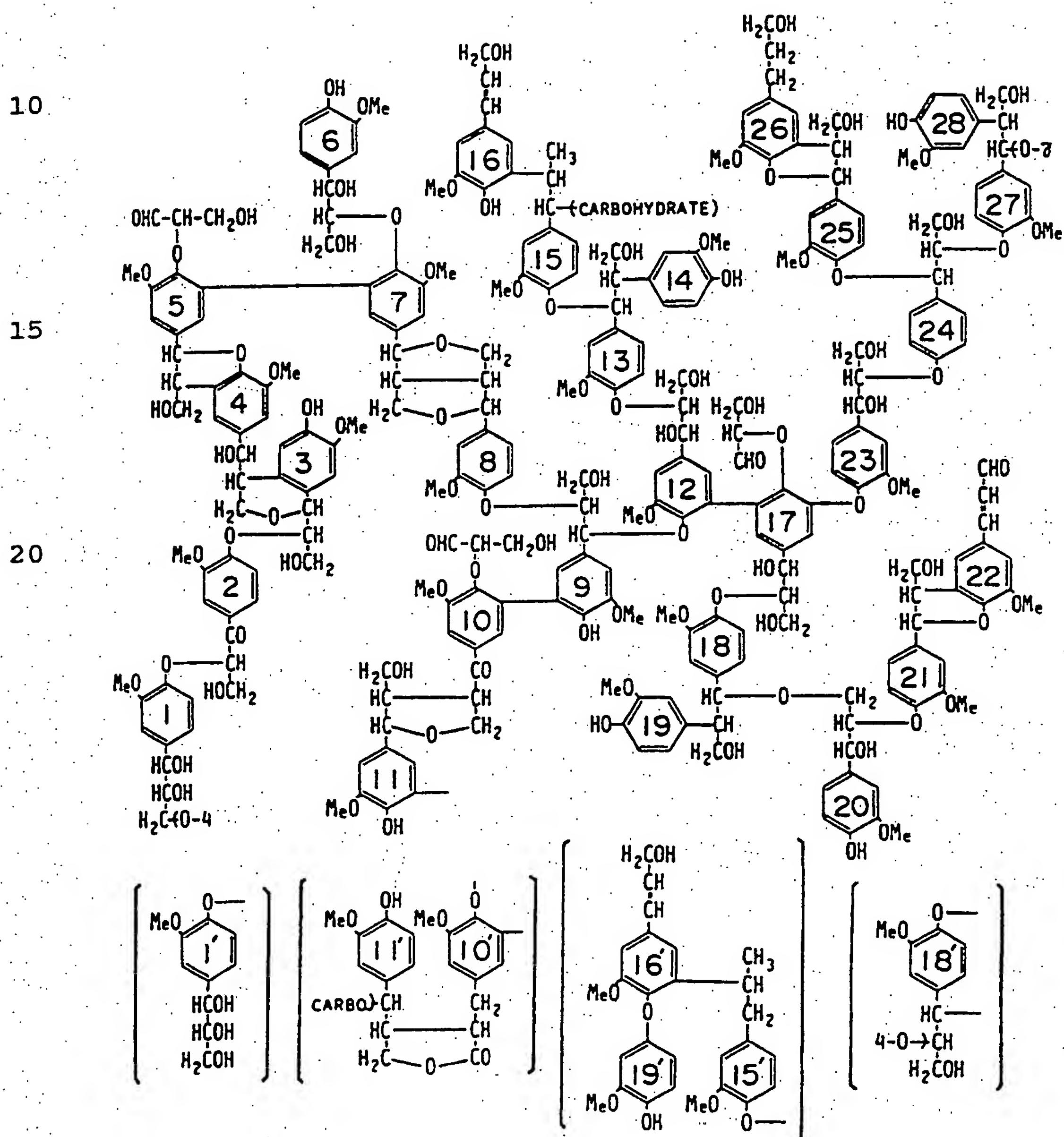
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(5)

(6)

A structure model of softwood lignin is described in Higuchi, Biosynthesis and Biodegradation of Wood Components, Wood Research Institute, Kyoto, Japan, 1985, page 143, and set forth below to show the variety of ring substitutions present in lignin.



The composition of lignin varies for different kinds of lignins. The lignin of hardwoods such as beech, for example, is composed of approximately equal amounts of guaiacyl- and syringylpropane units connected by linkages 5 similar to those found in spruce lignin. Grass lignin, such as bamboo lignin, is considered to be composed of guaiacyl-, syringyl-, and p-hydroxyphenyl.

The biodegradable azo dyes of the present invention include an azo group having a nitrogen atom 10 linked to an aromatic ring, in which the ring has a lignin-like substitution pattern. As used herein, the term "lignin-like substitution pattern" refers to a ring having substituents which provide a lignin-like structure. In its simplest embodiments, the lignin-like substitution 15 pattern provides guaiacyl or syringyl units connected by a nitrogen linkage to the remainder of the azo dye. The present inventors have found that biodegradability is especially enhanced by providing a lignin-like aromatic ring in which a first ring substituent R1 is selected from 20 the group consisting of hydroxy, lower alkoxy or amino, and a second substituent R2 is selected from the group consisting of lower alkyl, lower alkoxy and halogen. In especially preferred embodiments, a third ring substituent 25 R3 is selected from the group consisting of lower alkyl, lower alkoxy and halogen.

It is preferred, although not necessary, that the azo dye be a fully conjugated system. In particular embodiments, the dye includes a plurality of azo groups having nitrogen atoms linked to aromatic rings such that 30 the compound is a fully conjugated system. Diazo or triazo compounds, for example, would provide such a fully conjugated system. Such fully conjugated systems are both brighter and more susceptible to degradation. However, some less than fully conjugated dyes (such as C.I. direct 35 red 75; C.I direct orange; C.I. direct red 250; and C.I. direct yellow 27) may also be modified by adding lignin-like moieties to make them more biodegradable.

- 25 -

Modification of a portion of the dye molecule will at least make that part of the molecule more degradable, and may as a result make the entire molecule more degradable.

The aromatic ring having the lignin-like substitution pattern can be benzyl, naphthyl or other aromatic structures. A naphthyl ring is shown in azo dye 19. The dye may preferably include a sulfonic acid group to increase solubility of the dye. The sulfonic acid group may be present on either the lignin-like ring (as in dyes 18 or 19) or elsewhere in the molecule.

In particular embodiments, R2 is ortho to R1. In other embodiments, R1 is hydroxy while R2 is a lower alkoxy, such as methoxy, or a lower alkyl group such as methyl or ethyl. In preferred embodiments wherein R1 is hydroxy, R2 may preferably be halogen, such as fluorine or chlorine.

The azo dyes of the present invention preferably include at least one sulfonic acid group, on either the lignin-like ring or somewhere else in the molecule, to increase the solubility of the azo compound. This solubility is important to some dye applications.

In those embodiments in which R2 is ortho to R1, R2 may preferably be lower alkyl, lower alkoxy or halogen.

Several embodiments of the invention have been found to be particularly suitable for significant degradation by Phanerochaete. These embodiments include the following azo dyes: 4-dimethylamino-azobenzene-4'-sulfonic acid, 4-diethylamino-azobenzene-4'-sulfonic acid, 3-methyl-4-hydroxy-azobenzene-4'-sulfonic acid, 3-methoxy-4-hydroxy-azobenzene-4'-sulfonic acid, 3-chloro-4-hydroxy-azobenzene-4'-sulfonic acid, 3-sec-butyl-4-hydroxy-azobenzene-4'-sulfonic acid, 2-hydroxy-5-methyl-azobenzene-4'-sulfonic acid, 2-hydroxy-5-ethyl-azobenzene-4'-sulfonic acid, 3,5-dimethyl-4-hydroxy-azobenzene-4'-sulfonic acid, 3,5-dimethoxy-4-hydroxy-azobenzene-4'-sulfonic acid, 3,5-difluoro-4-hydroxy-azobenzene-4'-sulfonic acid, 2-hydroxy-4,5-dimethyl-azobenzene-4'-

- 26 -

sulfonic acid, 2-hydroxy-3-methoxy-5-methyl-azobenzene-4'-sulfonic acid.

Several especially preferred embodiments are very completely degraded by Phanerochaete, and include 4-dimethylamino-azobenzene-4'-sulfonic acid, 3-methyl-4-hydroxy-azobenzene-4'-sulfonic acid, 3-methoxy-4-hydroxy-azobenzene-4'-sulfonic acid, 3-chloro-4-hydroxy-azobenzene-4'-sulfonic acid, 3-sec-butyl-4-hydroxy-azobenzene-4'-sulfonic acid, 3,5-dimethoxy-4-hydroxy-azobenzene-4'-sulfonic acid, 3,5-difluoro-4-hydroxy-azobenzene-4'-sulfonic acid, 2-hydroxy-4,5-dimethyl-azobenzene-4'-sulfonic acid, 2-hydroxy-3-methoxy-5-methyl-azobenzene-4'-sulfonic acid.

Other compounds show some biodegradability when cultured with the Streptomyces strains of the present invention. Examples of such compounds having a higher degree of biodegradation with Streptomyces are the following: 3-methyl-4-hydroxy-azobenzene-4'-sulfonic acid, 3-methoxy-4-hydroxy-azobenzene-4'-sulfonic acid, 3-chloro-4-hydroxy-azobenzene-4'-sulfonic acid, 3-sec-butyl-4-hydroxy-azobenzene-4'-sulfonic acid, 3,5-dimethyl-4-hydroxy-azobenzene-4'-sulfonic acid, 3,5-dimethoxy-4-hydroxy-azobenzene-4'-sulfonic acid, and 2-hydroxy-3-methoxy-5-methyl-azobenzene-4'-sulfonic acid and 4-diethylamino-azobenzene-4'-sulfonic acid.

Especially well degraded dyes with Streptomyces include 3-methyl-4-hydroxy-azobenzene-4'-sulfonic acid, 3-methoxy-4-hydroxy-azobenzene-4'-sulfonic acid, 3-sec-butyl-4-hydroxy-azobenzene-4'-sulfonic acid, 3,5-dimethyl-4-hydroxy-azobenzene-4'-sulfonic acid, 3,5-dimethoxy-4-hydroxy-azobenzene-4'-sulfonic acid. These dyes were significantly degraded by Streptomyces, that is degraded more than about 10%.

Particularly high Streptomyces degradation is observed when R1 is hydroxy, and R2 and R3 are methyl, particularly when the methyls are both ortho to the hydroxy. Similarly, high Streptomyces degradation is seen

- 27 -

when R1 is hydroxy and R2 and R3 are both methoxy, particularly if both R2 and R3 are ortho to R1. Other Streptomyces degradable compounds include those in which R1 is hydroxy, R2 is methyl and R3 is methoxy, especially 5 wherein R2 and R3 are both ortho to R1.

The present invention also includes a biodegradable composition containing an azo dye having a nitrogen atom linked to an aromatic ring with a lignin-like substitution pattern, wherein the composition also 10 includes a microbe capable of degrading the dye. The ring has a first substituent R1 selected from the group consisting of hydroxy, alkoxy and amino, and a second substituent R2 selected from the group consisting of hydrogen, lower alkoxy, lower alkyl and hydrogen. The 15 amino in preferred embodiments is secondary amine.

In particularly preferred embodiments of the composition, the microbe is Phanerochaete chrysosporium. Subspecies of the azo dye that are particularly useful in such a composition include those wherein R1 is hydroxy, 20 particularly if R2 is a lower alkoxy, lower alkyl or halogen.

In yet other embodiments of the composition, the microbe is a Streptomyces, for example S.rochei, S.chromofuscus, S.diastaticus, S.viridosporus, or 25 S.badius. Particularly useful strains of Streptomyces have been found to be S.rochei A10, A14, S.chromofuscus A11, A20, S.diastaticus A12, A13, S.viridosporus T7A and S.badius 252. Several compounds have been found to be particularly biodegradable in combination with 30 Streptomyces. Such compounds include those in which R1 is hydroxy, particularly when R2 is ortho to the hydroxy. Enhanced biodegradability is also observed when the ring includes a third ring substituent R3 selected from the group consisting of lower alkyl and lower alkoxy. 35 Biodegradability is particularly high when R1 is a hydroxy para to the azo linkage, and R2 is ortho to the hydroxy.

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In such embodiments, R₂ is most preferably a lower alkoxy or lower alkyl.

In yet other embodiments of the invention, the biodegradability of xenobiotic dyes is increased by 5 introducing a lignin-like aromatic ring into a preexisting azo dye.

Persons skilled in the art will recognize that azo dyes, other than those specifically disclosed, are included in the scope of this invention. Other 10 microorganisms are also suitable for use in degrading these azo dyes. Soil microflora in general are a good source of additional microorganisms, which can be tested for biodegradative capacity as described in this specification. The dyes can also be degraded in soil 15 itself, which contains many species of organisms capable of degrading the lignin-like dyes of the present invention.

The present application describes certain strains of soil Streptomyces species which are particularly 20 effective at degrading the disclosed azo dyes. Such natural variability is expected, and is not evidence of any limitation of the method to use with particular strains of bacteria. Any person skilled in the art will be able to select bacteria from soil or elsewhere using 25 the biotransformation assays disclosed herein. Actual selection of individual biodegradative species and strains is not essential because a mixture of soil microflora contains the microorganisms sufficient for azo dye biotransformation.

30 Table 2 illustrates that higher concentrations of azo dyes are sometimes less effectively degraded by P.chrysosporium. Dye 3, for example, becomes more toxic to the organism at 300 ppm, in contrast to concentrations below 300 ppm. Dyes 4 and 5 do not exhibit a similar 35 degree of inhibition. In any case, toxic inhibition is not complete even at 300 ppm in sensitive organisms. Optimum concentrations of substrate are very specific to

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the substrate and organism of interest, and are subject to the kind of routine optimization known to those skilled in the art.

Having illustrated and described the principles
5 of the invention in several preferred embodiments, it
should be apparent to those skilled in the art that the
invention can be modified in arrangement and detail
without departing from such principles. We claim all
modifications coming within the spirit and scope of the
10 following claims.

- 30 -

We Claim:

1. A biodegradable dye compound comprising:
an azo group having a nitrogen atom linked to an
aromatic ring, the ring having a lignin-like substitution
5 pattern.
2. The dye of claim 1 wherein the lignin-like
substitution pattern is a syringyl or guaiacyl moiety.
3. The dye of claim 1 wherein the aromatic ring
has a first substituent R₁ selected from the group
10 consisting of hydroxy, lower alkoxy, or amino, and a
second substituent R₂ selected from the group consisting
of lower alkyl, lower alkoxy and halogen.
4. The dye of claim 3 further comprising a third
ring substituent R₃ selected from the group consisting of
15 lower alkyl, lower alkoxy, and halogen.
5. The dye of claim 3 further comprising a
plurality of azo groups having nitrogen atoms linked to
aromatic rings such that the compound is a fully
conjugated system.
- 20 6. The dye of claim 4 further comprising a
plurality of azo groups having nitrogen atoms linked to
aromatic rings such that the compound is a fully
conjugated system.
7. The dye of claim 3 wherein R₂ is ortho to R₁.
- 25 8. The dye of claim 5 wherein R₂ is ortho to R₁.
9. The dye of claim 3 wherein R₁ is hydroxy.
10. The dye of claim 9 wherein R₂ is a lower
alkoxy.
- 30 11. The dye of claim 9 wherein R₂ is methoxy.
12. The dye of claim 9 wherein R₂ is a lower
alkyl group.
13. The dye of claim 12 wherein R₂ is selected
from the group consisting of methyl and ethyl.
- 35 14. The dye of claim 9 wherein R₂ is halogen.
15. The dye of claim 14 wherein R₂ is selected
from the group consisting of fluorine and chlorine.

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16. The dye of claim 1 wherein the azo dye further includes at least one sulfonic acid group.

17. The dye of claim 7 wherein R₂ is lower alkyl.

5 18. The dye of claim 7 wherein R₂ is lower alkoxy.

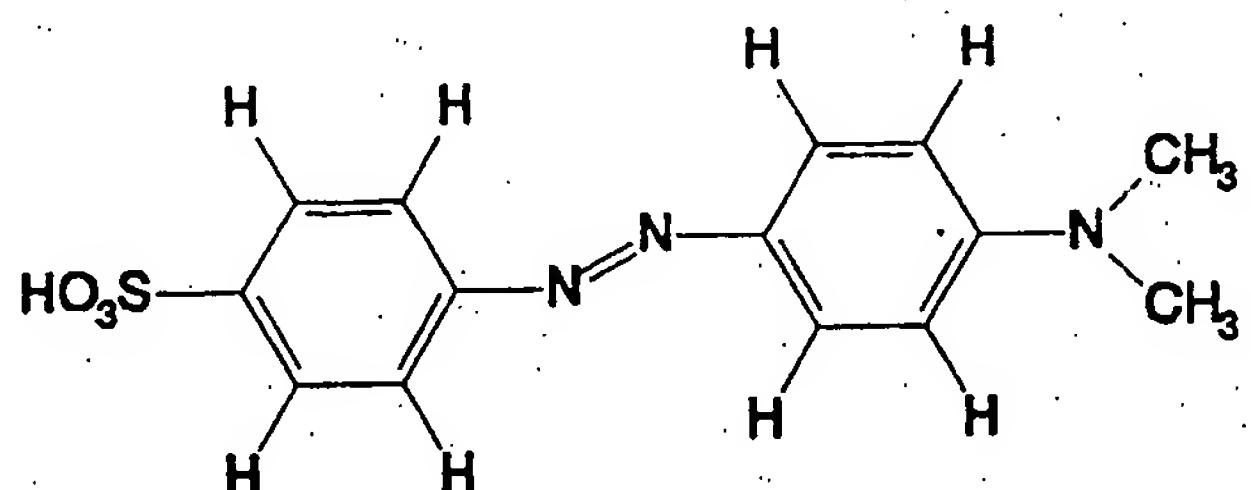
19. The dye of claim 7 wherein R₂ is halogen.

20. The dye of claim 1 wherein said compound is selected from the group consisting of

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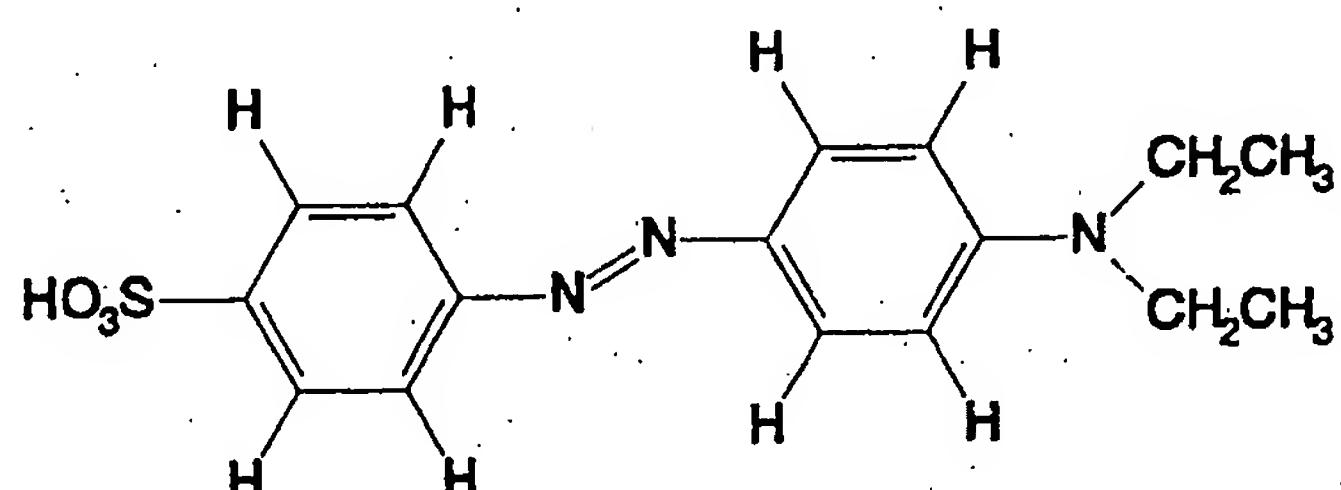
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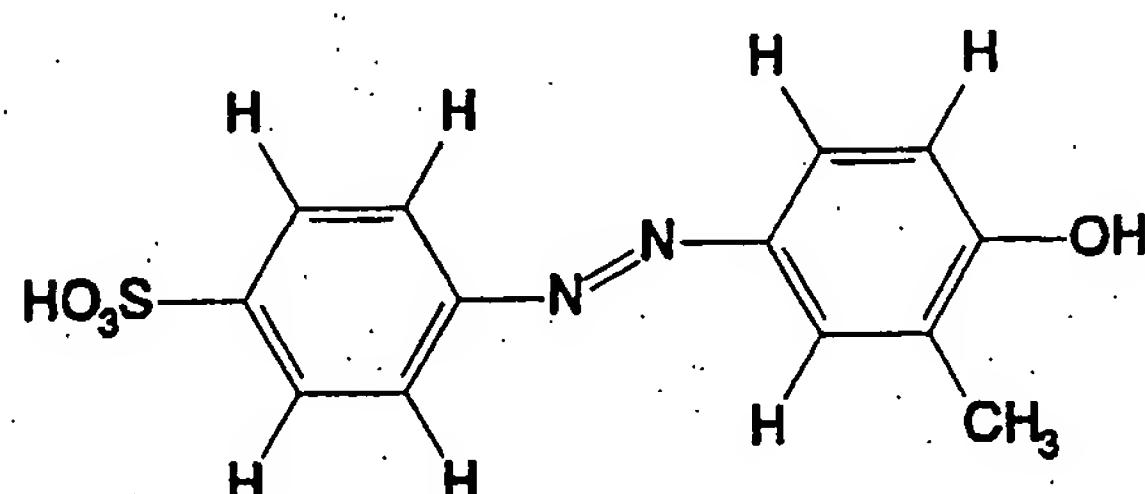
20 4-diethylamino-azobenzene-4'-sulfonic acid,



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3-methyl-4-hydroxy-azobenzene-4'-sulfonic acid,

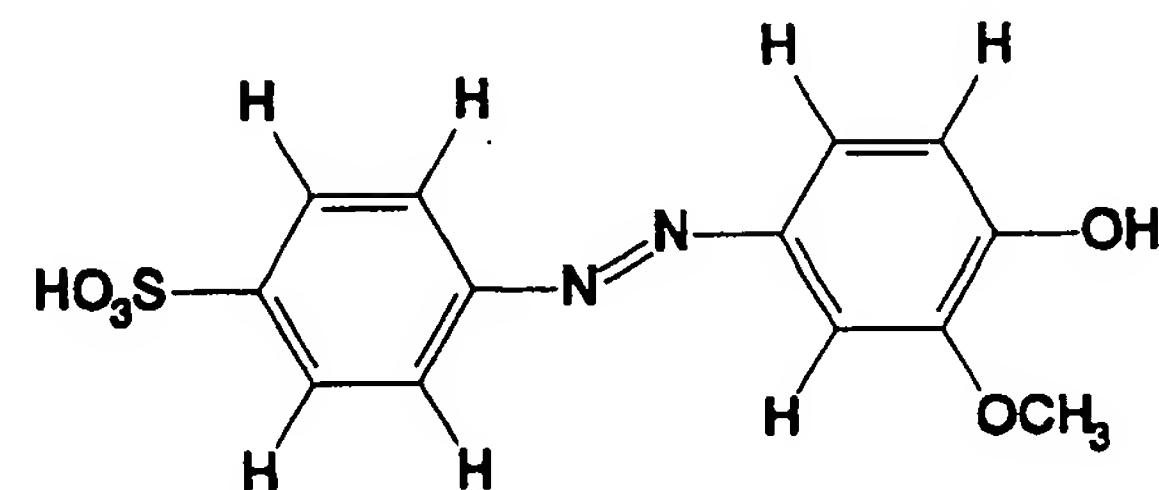
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- 31A -

3-methoxy-4-hydroxy-azobenzene-4'-sulfonic acid,

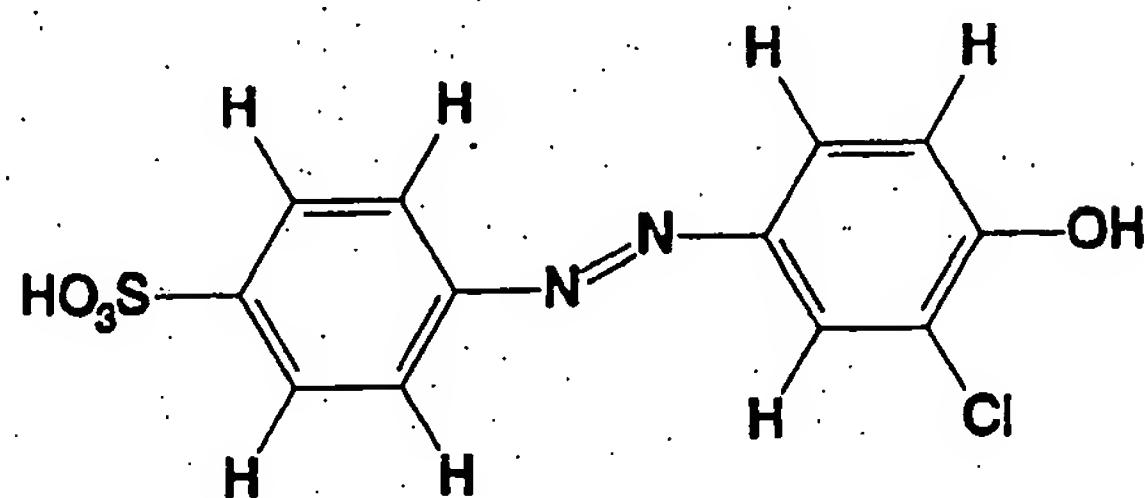
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- 32 -

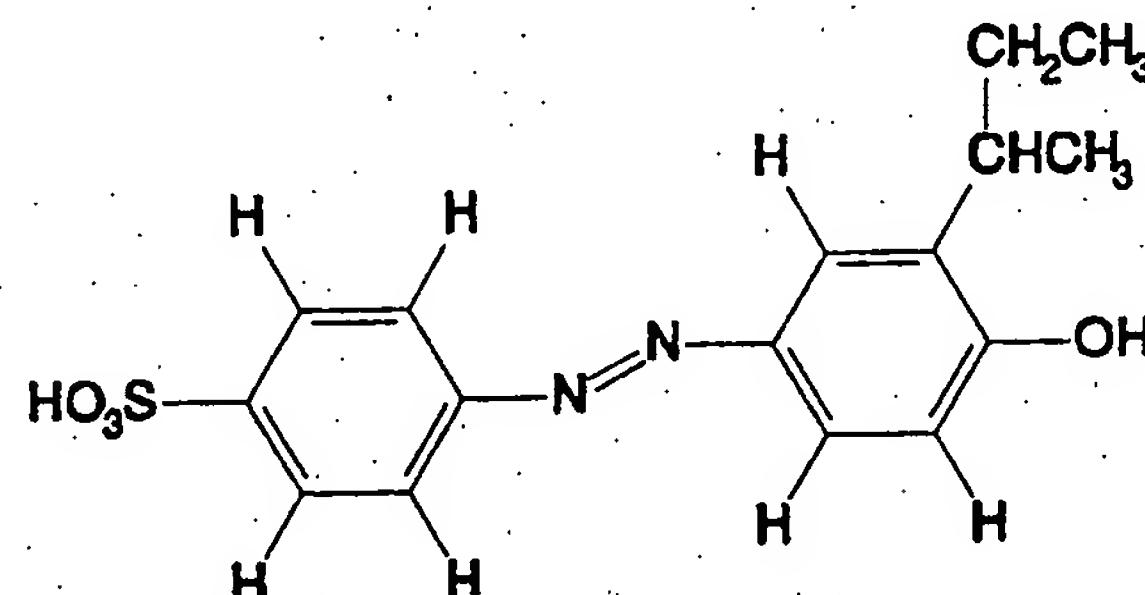
3-chloro-4-hydroxy-azobenzene-4'-sulfonic acid,

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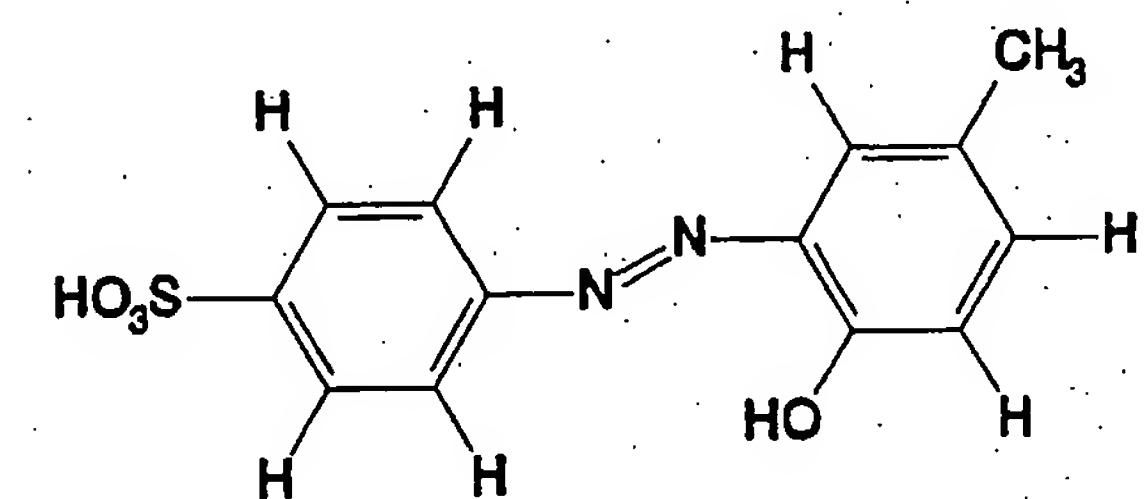
10 3-sec-butyl-4-hydroxy-azobenzene-4'-sulfonic acid,

15



20 2-hydroxy- 5-methyl-azobenzene-4'-sulfonic acid,

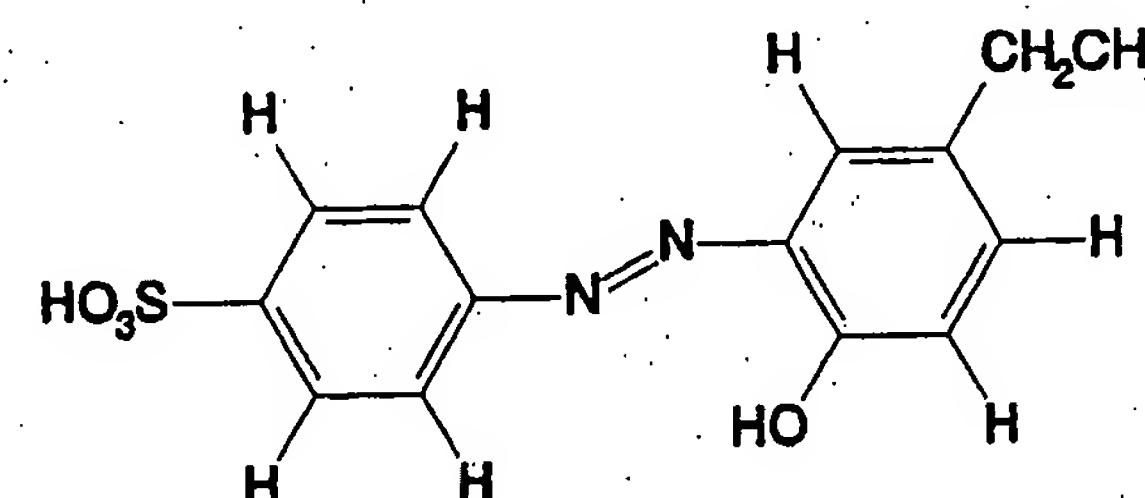
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2-hydroxy-5-ethyl-azobenzene-4'-sulfonic acid,

30

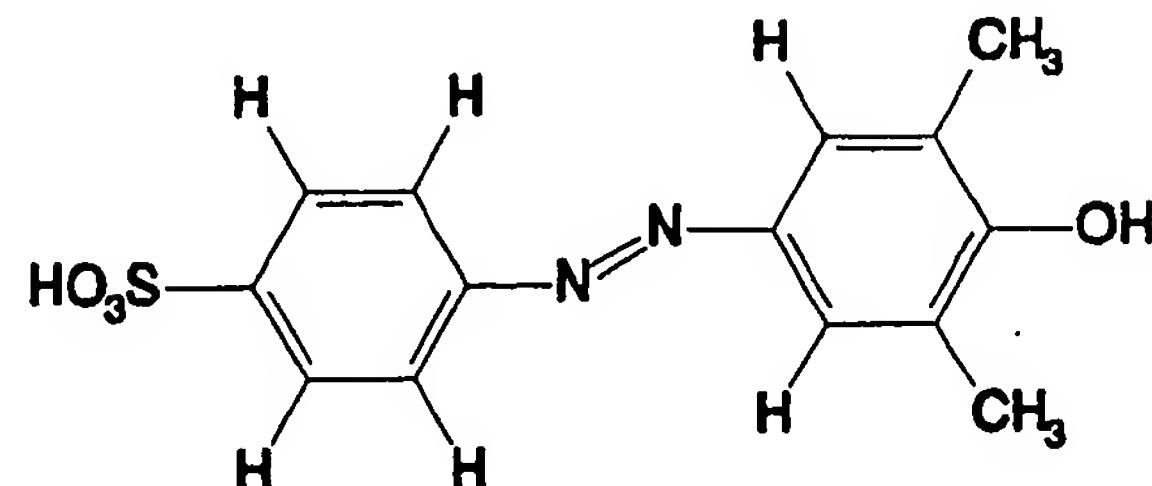
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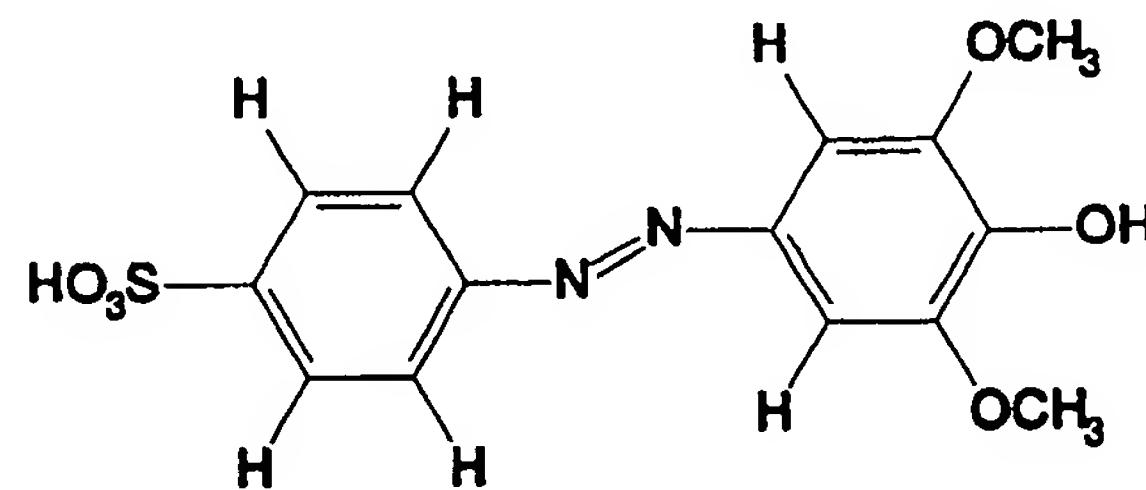
- 32A -

3,5-dimethyl-4-hydroxy-azobenzene-4'-sulfonic acid,

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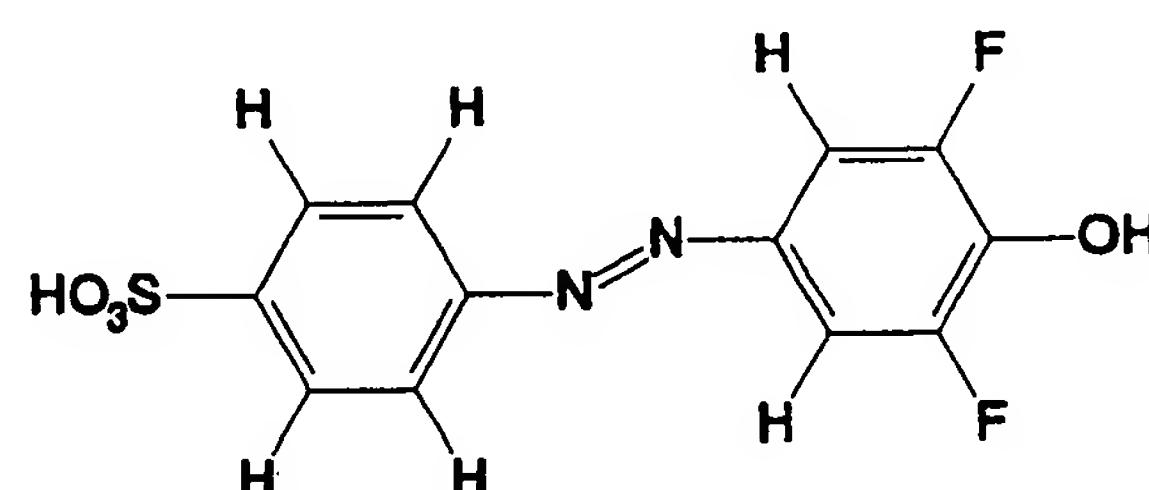
**10 3,5-dimethoxy- 4-hydroxy-azobenzene-4'-sulfonic acid,**

15



20

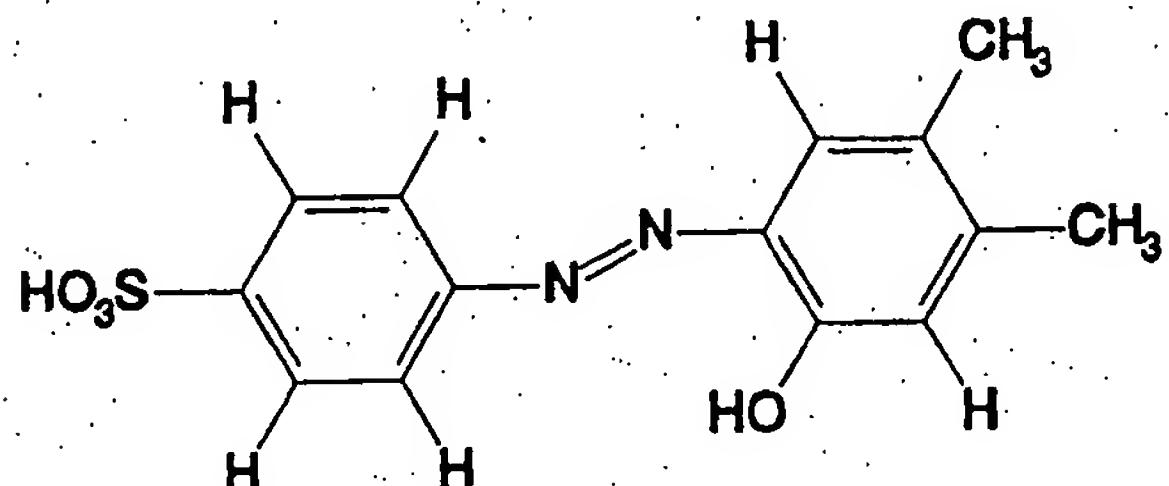
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- 33 -

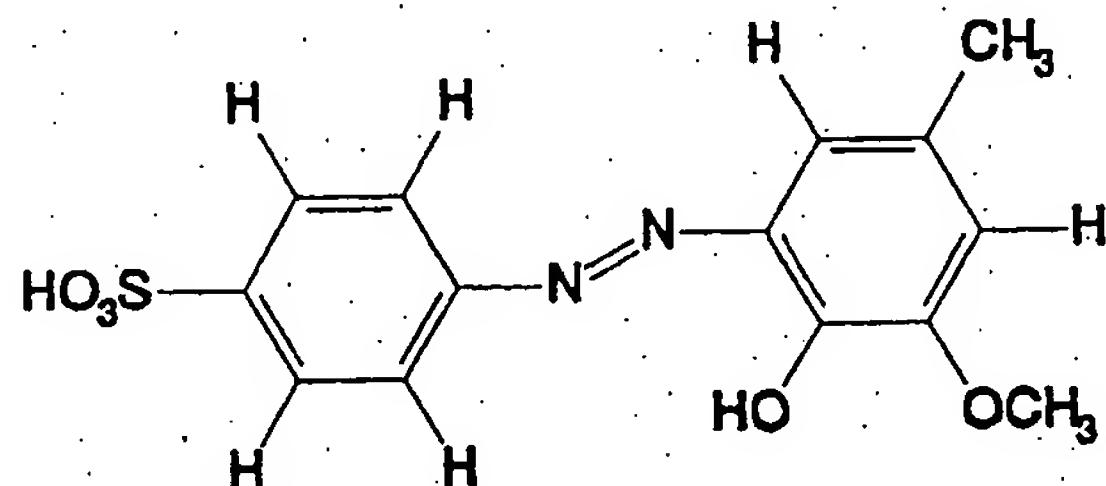
2-hydroxy-4,5-dimethyl-azobenzene-4'-sulfonic acid,

5



10 2-hydroxy-3-methoxy-5-methyl-azobenzene-4'-sulfonic acid.

15



21. The dye compound of claim 1 wherein said
20 compound is selected from the group consisting of 3-
methyl-4-hydroxy-azobenzene-4'-sulfonic acid, 3-methoxy-4-
hydroxy-azobenzene-4'-sulfonic acid, 3-chloro-4-hydroxy-
azobenzene-4'-sulfonic acid, 3-sec-butyl-4-hydroxy-
azobenzene-4'-sulfonic acid, 3,5-dimethyl-4-hydroxy-
25 azobenzene-4'-sulfonic acid, 3,5-dimethoxy-4-hydroxy-
azobenzene-4'-sulfonic acid, and 2-hydroxy-3-methoxy-5-
methyl-azobenzene-4'-sulfonic acid.

22. The dye compound of claim 4 wherein R₁ is
hydroxy, R₂ and R₃ are methyl.

30 23. The dye compound of claim 22 wherein R₂ and
R₃ are both ortho to R₁.

24. The dye compound of claim 4 wherein R₁ is
hydroxy and R₂ and R₃ are methoxy.

25. The dye compound of claim 24 wherein both R₂
35 and R₃ are ortho to R₁.

26. The dye compound of claim 4 wherein R₁ is
hydroxy and R₂ is methyl and R₃ is methoxy.

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27. The dye compound of claim 26 wherein R₂ and R₃ are both ortho to R₁.

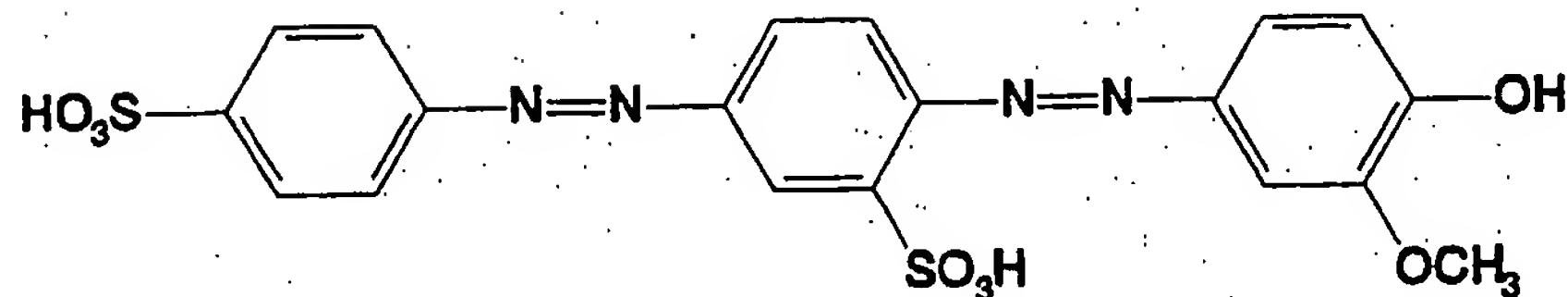
28. The dye compound of claim 4 wherein R₂ and R₃ are both halogen.

5 29. The dye compound of claim 28 wherein R₂ and R₃ are both ortho to R₁.

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30. The dye compound of claim 3 wherein said compound is

5



31. A biodegradable composition comprising:
10 an azo dye having a nitrogen atom linked to an aromatic ring, the ring having a lignin-like substitution pattern; and
a microbe capable of degrading said dye.

32. The composition of claim 30 wherein the ring
15 has a first substituent R₁ selected from the group consisting of hydroxy, alkoxy and amino, and a second substituent R₂ selected from the group consisting of hydrogen, lower alkoxy, lower alkyl and halogen.

33. The composition of claim 32 wherein the
20 microbe is a white-rot fungus Phanerochaete chrysosporium.

34. The composition of claim 33 wherein R₁ is hydroxy.

35. The composition of claim 34 wherein R₂ is a lower alkoxy.

25 36. The dye of claim 34 wherein R₂ is a lower alkyl group.

37. The composition of claim 34 wherein R₂ is halogen.

38. The composition of claim 32 wherein the
30 compound is selected from the group consisting of 4-dimethylamino-azobenzene-4'-sulfonic acid, 4-diethylamino-azobenzene-4'sulfonic acid, 3-methyl-4-hydroxy-azobenzene-4'-sulfonic acid, 3-methoxy-4-hydroxy-azobenzene-4'-sulfonic acid, 3-chloro-4-hydroxy-azobenzene-4'-sulfonic acid, 3-sec-butyl-4-hydroxy-azobenzene-4'-sulfonic acid, 3,5-dimethoxy-4-hydroxy-azobenzene-4'-sulfonic acid, 3,5-difluoro-4-hydroxy-azobenzene-4-sulfonic acid, 3,5-

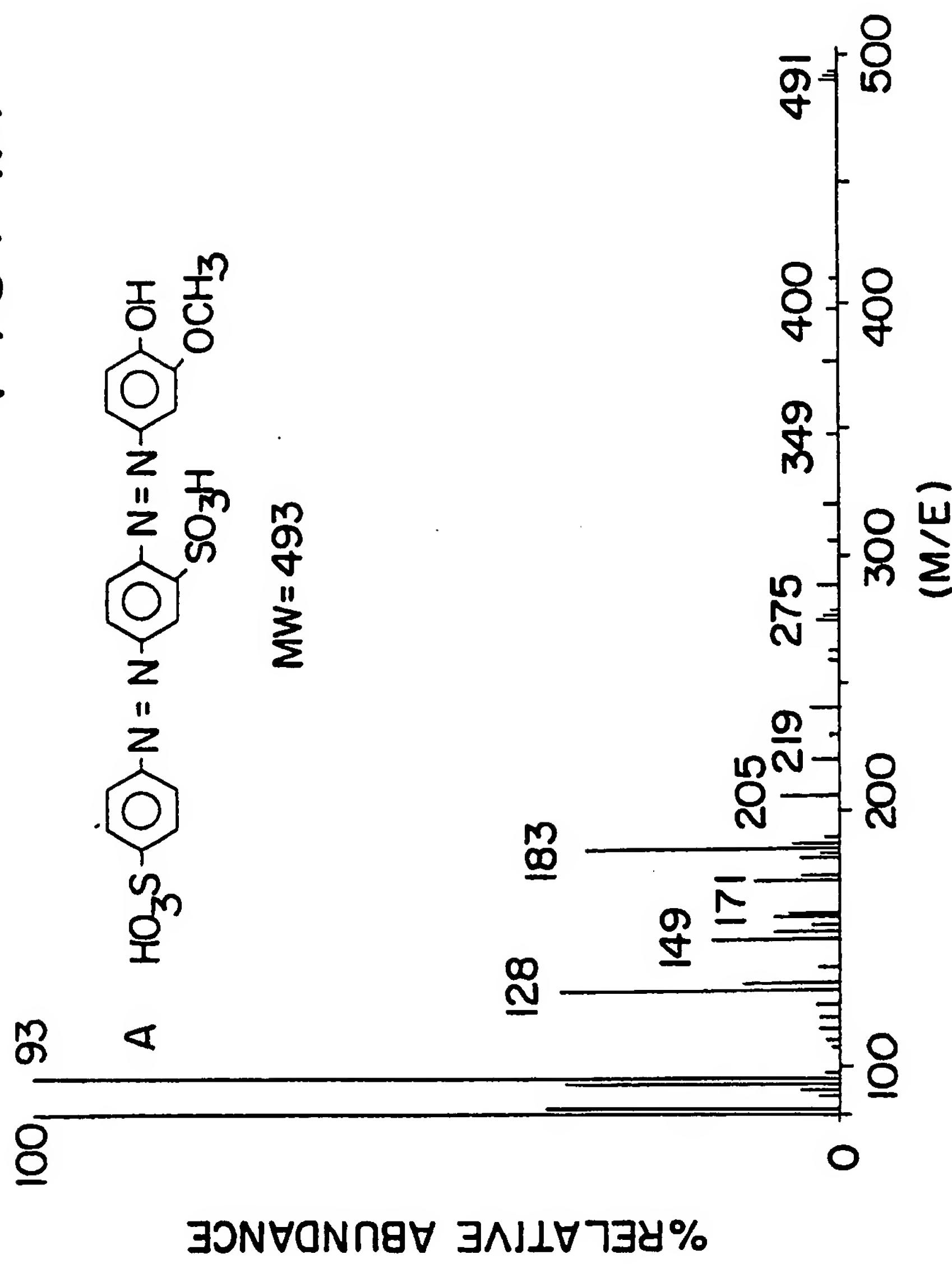
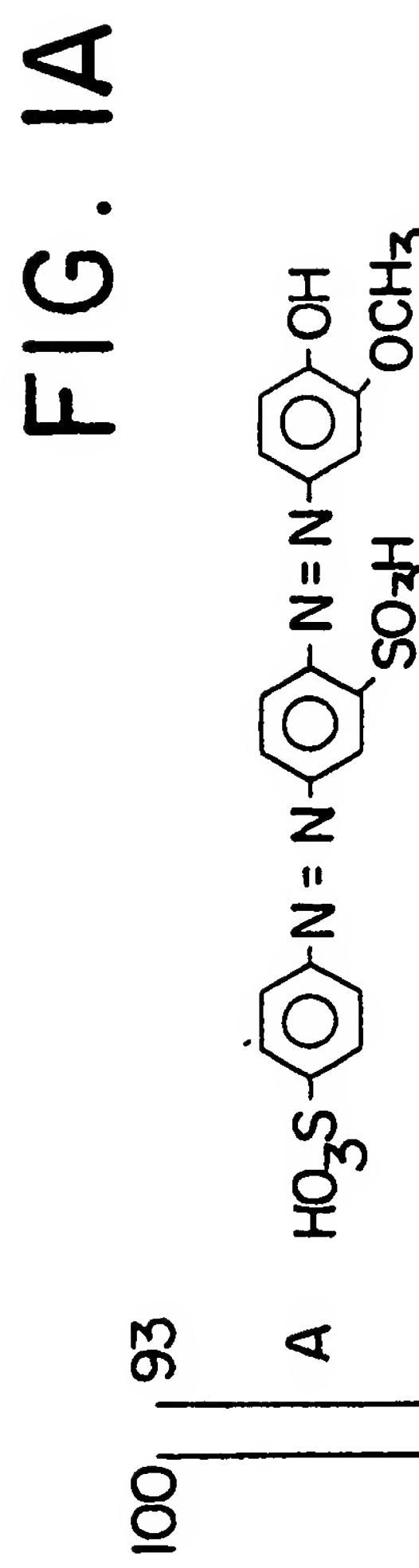
- 34A -

dimethyl-4-hydroxy-azobenzene-4'-sulfonic acid, 2-hydroxy-
4,5-dimethyl-azobenzene-4'-sulfonic acid, 2-hydroxy-3-
methoxy-5-methyl-azobenzene-4'-sulfonic acid, and 4-(3-
methoxy-4-hydroxyphenylazo)-azobenzene-3,4'-disulfonic
5 acid.

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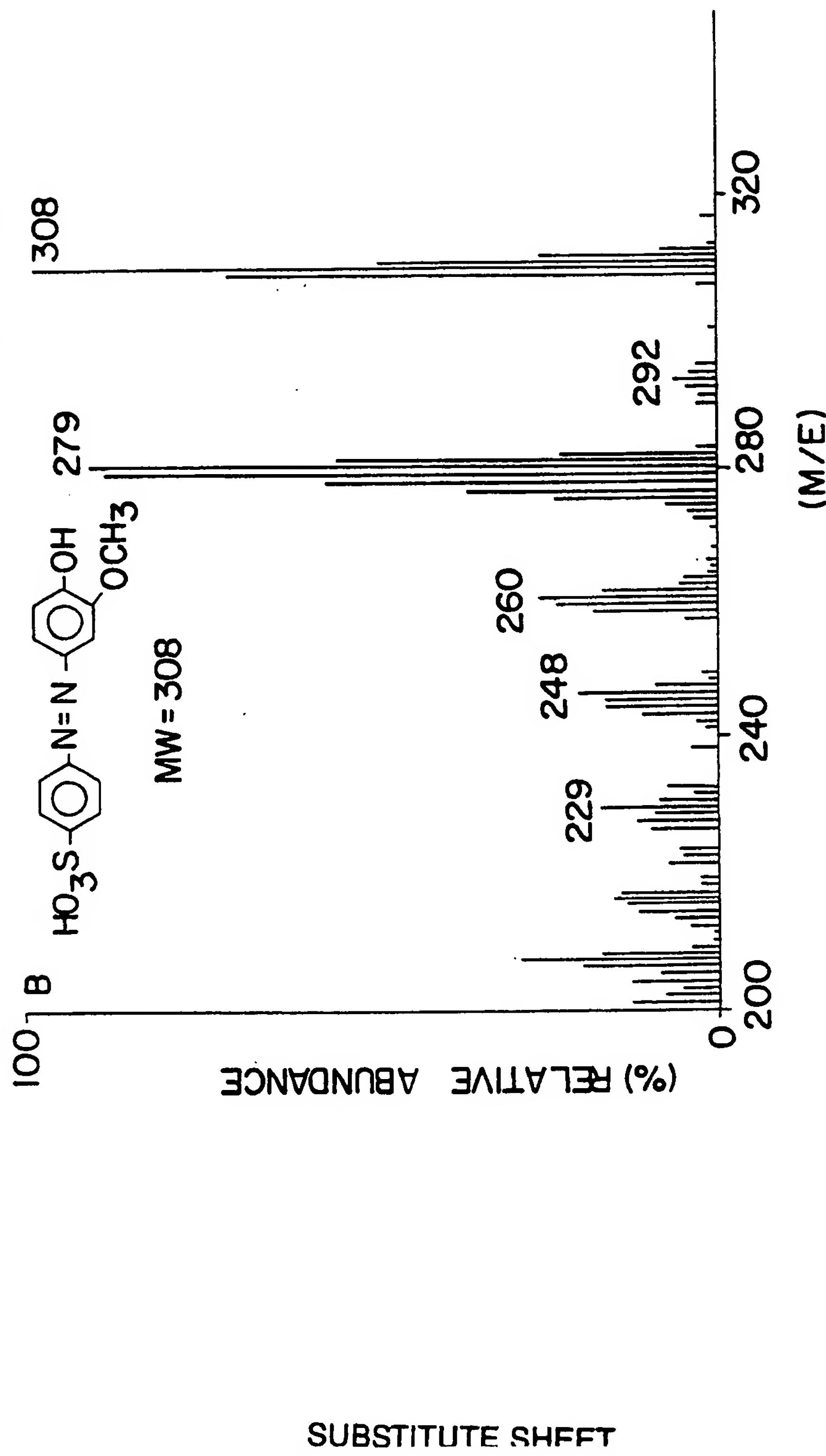
39. The composition of claim 32 wherein the microbe is a Streptomyces and R₁ is hydroxy.
40. The composition of claim 39 wherein R₂ is ortho to R₁.
- 5 41. The composition of claim 40 wherein the dye further comprises a third ring substituent R₃ selected from the group consisting of lower alkyl and lower alkoxy.
42. The composition of claim 39 wherein the Streptomyces is selected from the group consisting of 10 S.rochei, S.badius, S.chromofuscus, and S.diastaticus.
43. The composition of claim 39 wherein the hydroxy is para to the azo linkage, and R₂ is ortho to the hydroxy.
- 15 44. The composition of claim 40 wherein R₂ is a lower alkoxy.
45. The dye of claim 40 wherein R₂ is a lower alkyl group.
46. The composition of claim 31 wherein said amino is a secondary amine.
- 20 47. The composition of claim 39 wherein the Streptomyces is a vanillic acid degrading Streptomyces.
48. A method of increasing the biodegradability of xenobiotic azo dyes having a nitrogen atom linked to an aromatic ring, the method comprising providing a lignin-like substitution pattern on the aromatic ring.
- 25 49. The method of claim 48 further comprising the step of exposing the dye to soil microflora.
50. The composition of claim 31 wherein the microbe is a member of the soil microflora.

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FIG. I B



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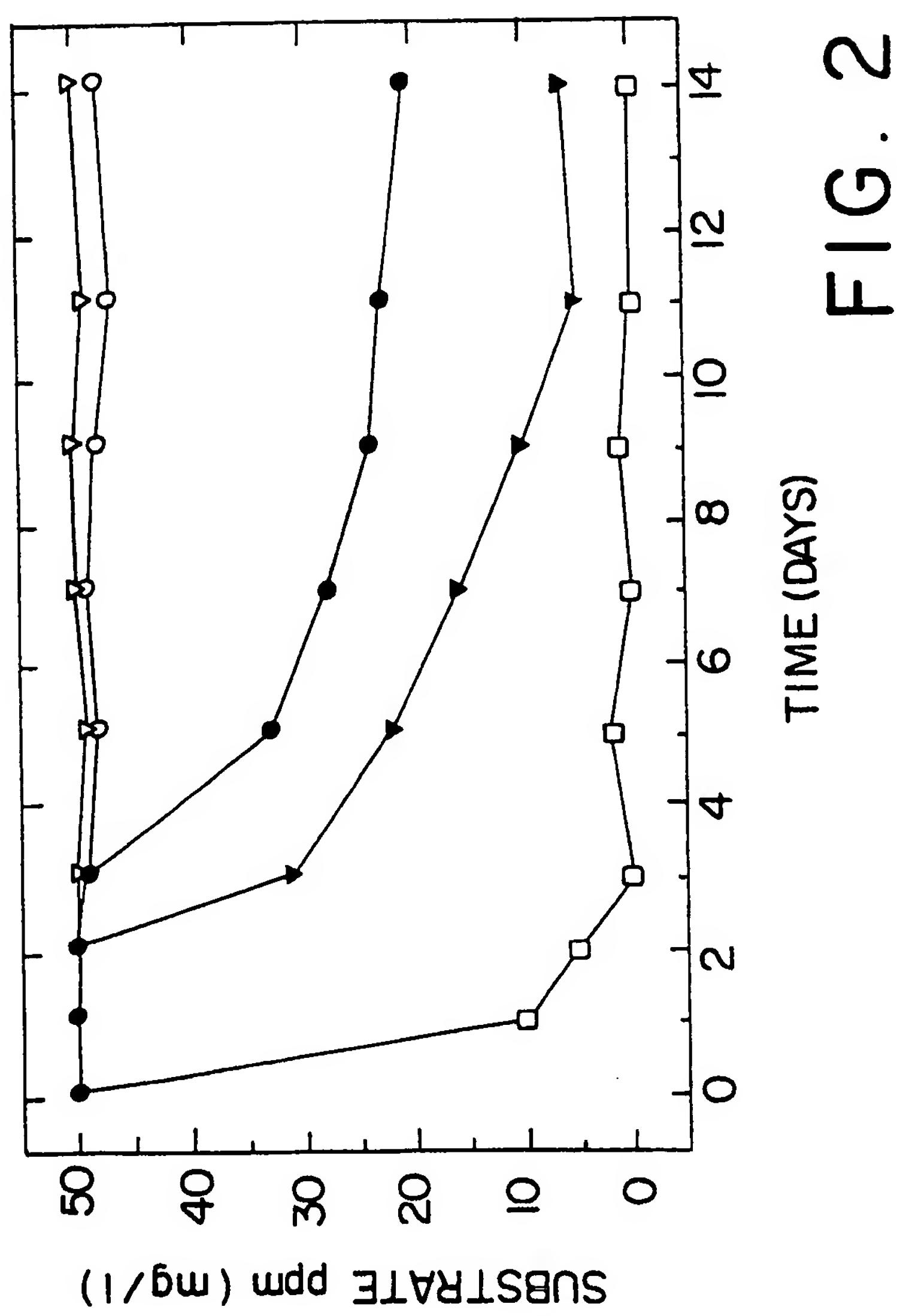
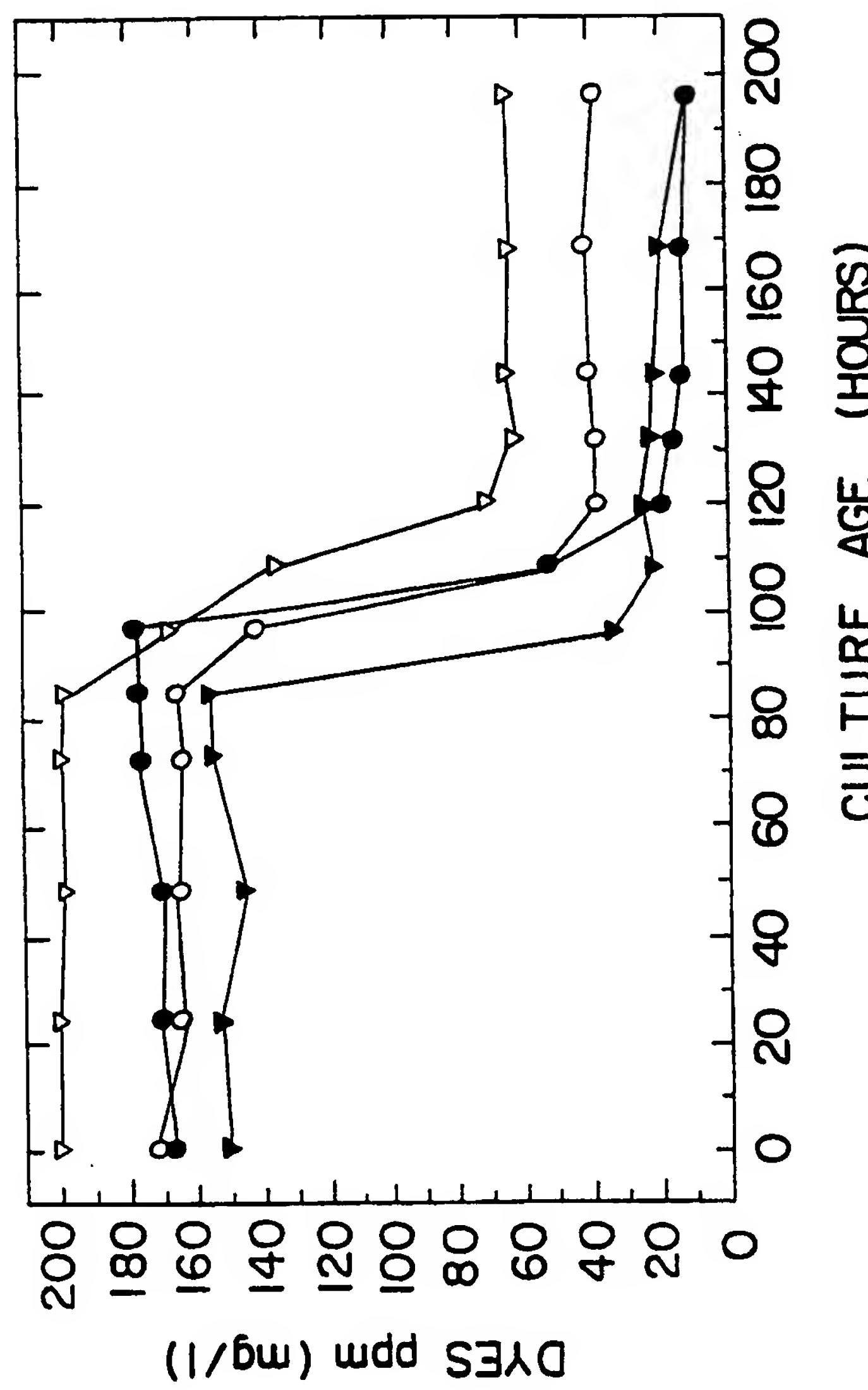


FIG. 2

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FIG. 3



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FIG. 4A

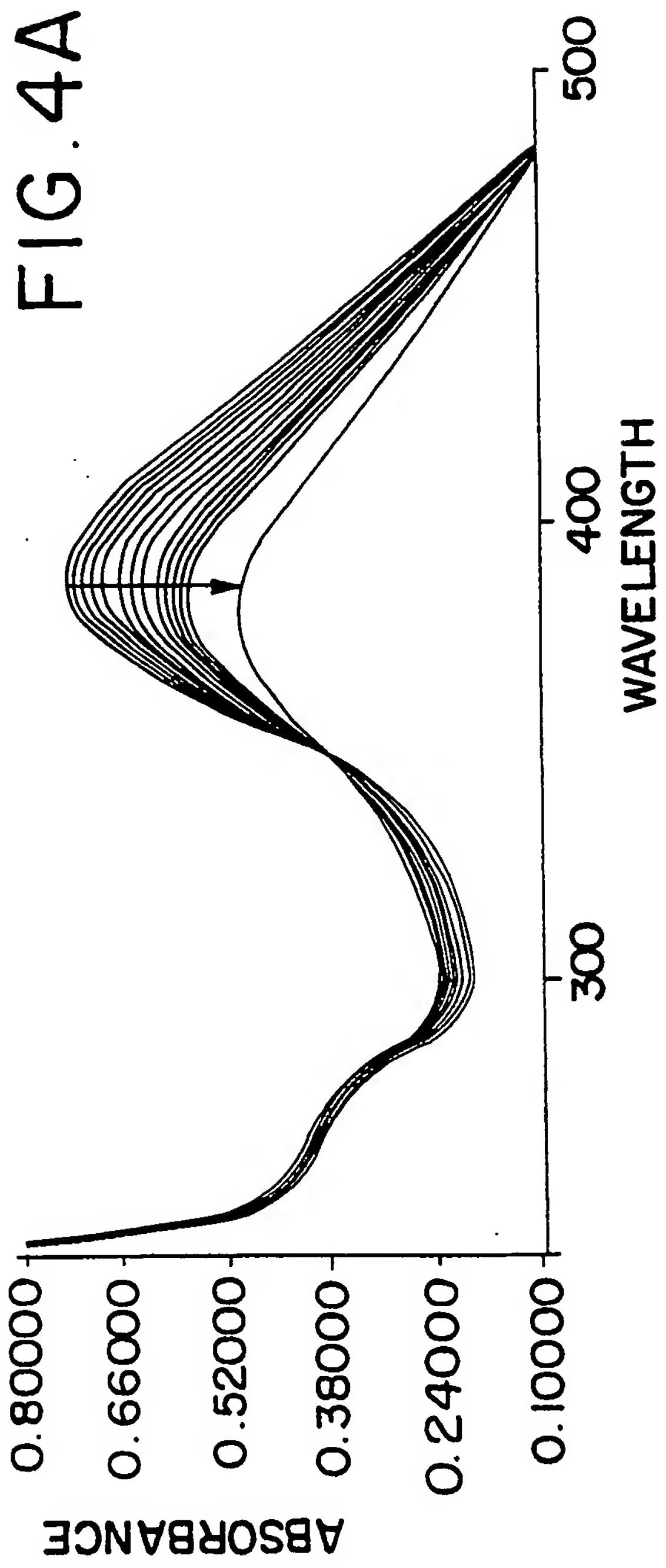
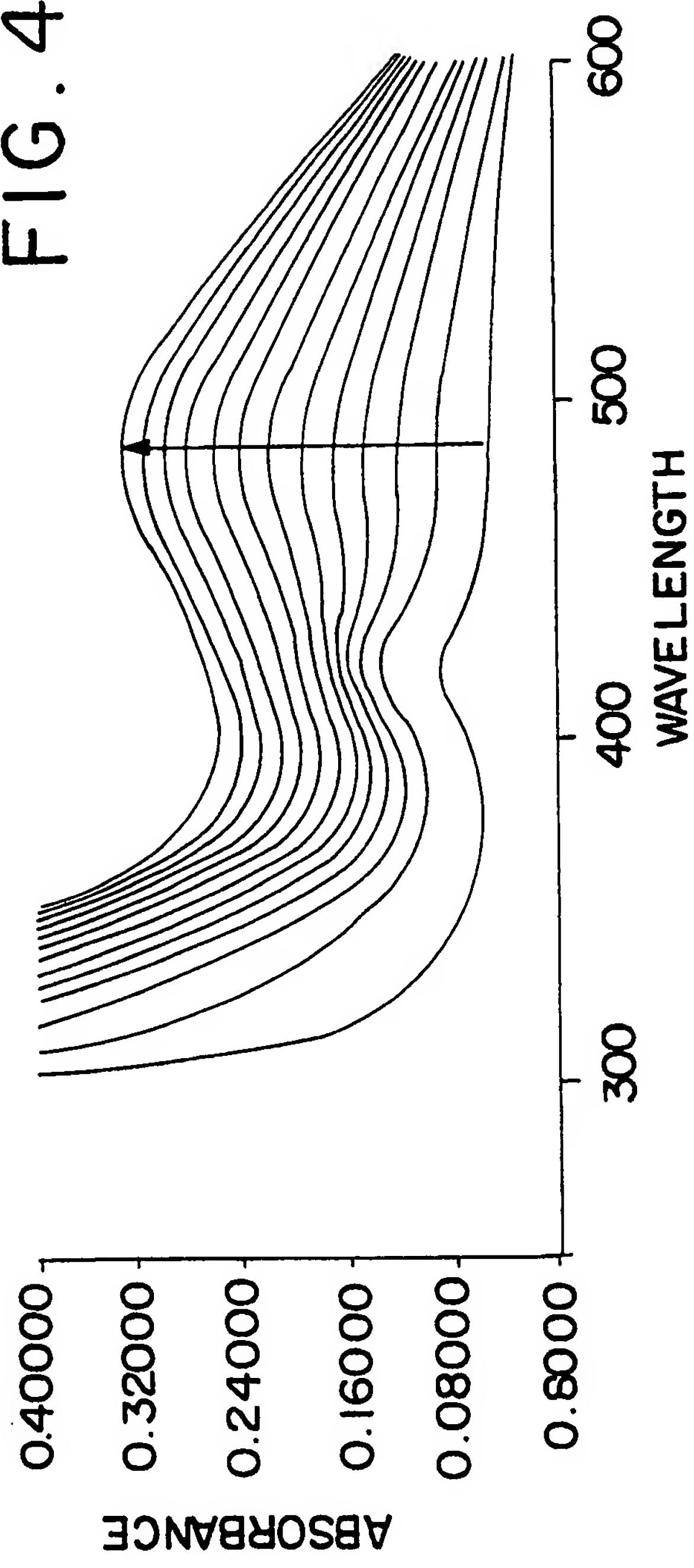


FIG. 4B



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FIG. 4 C

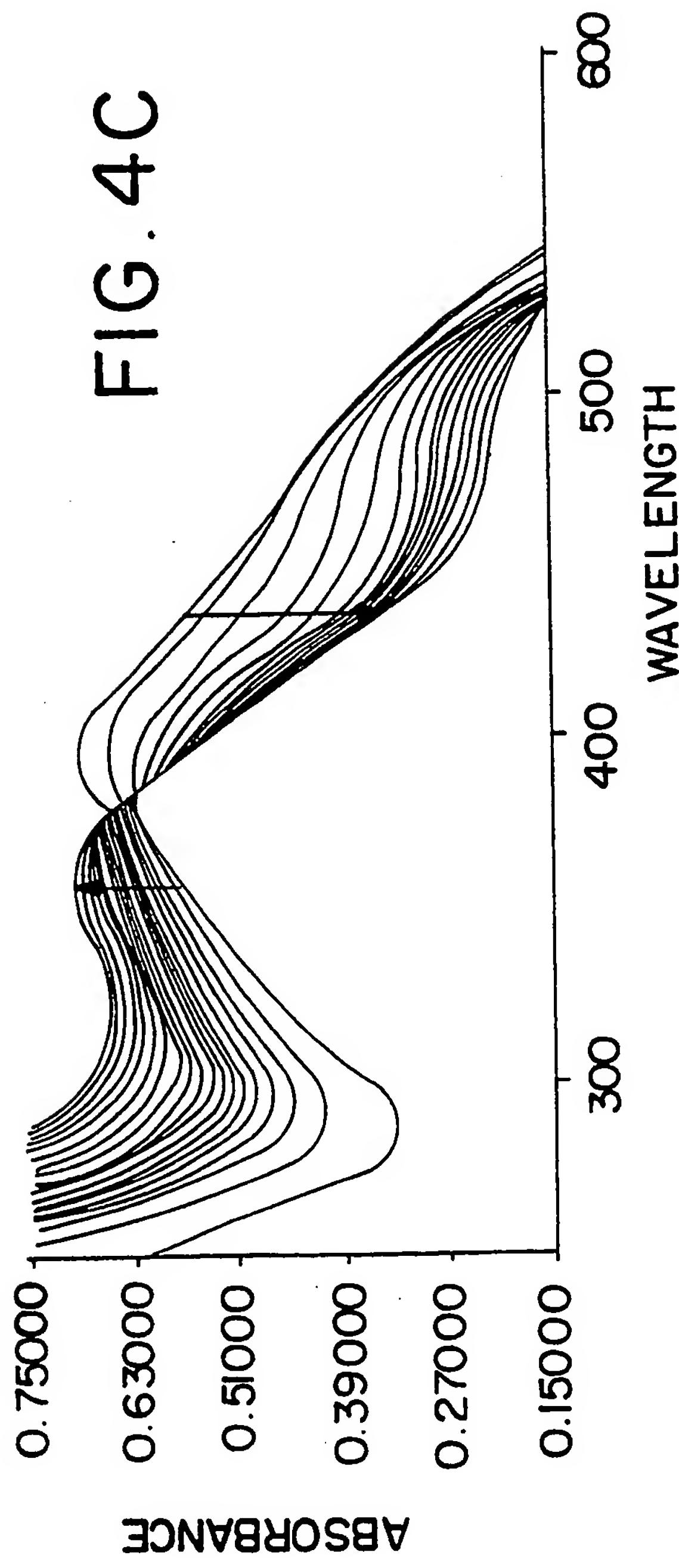
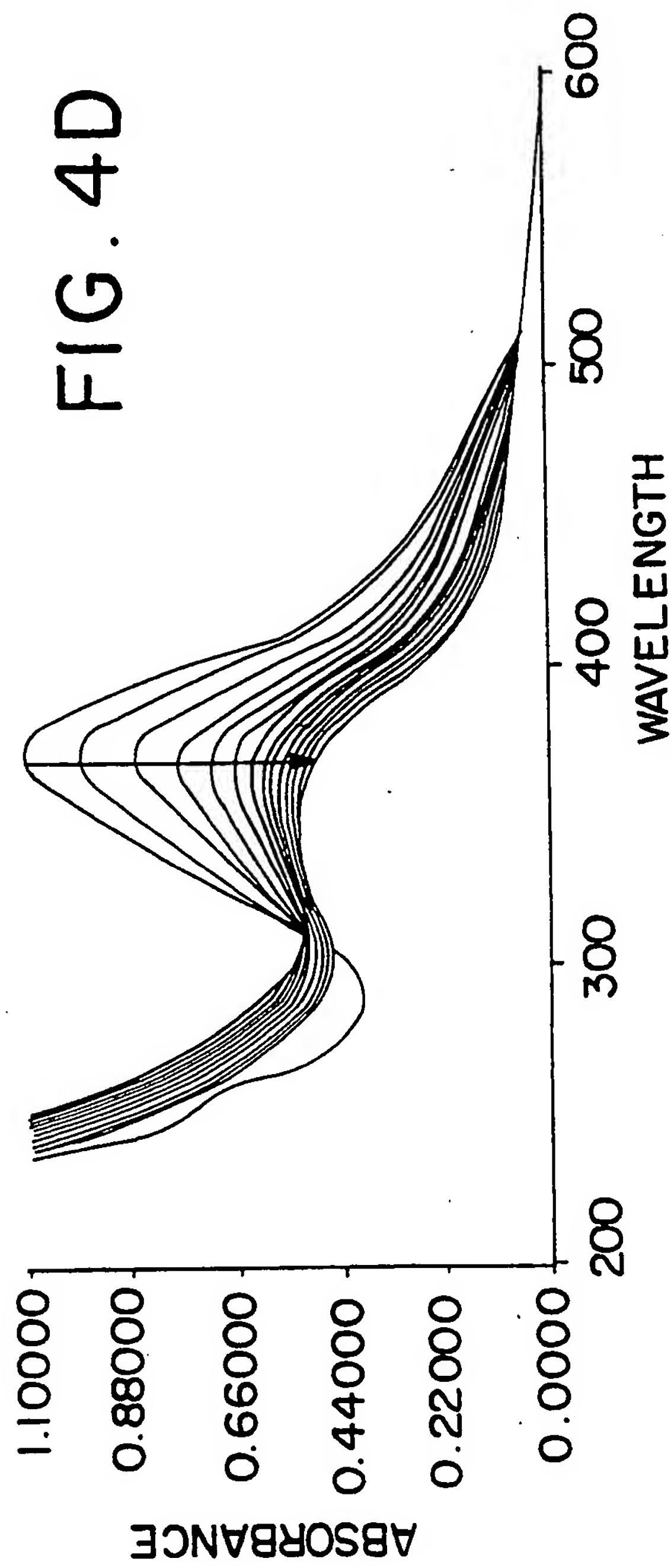


FIG. 4 D



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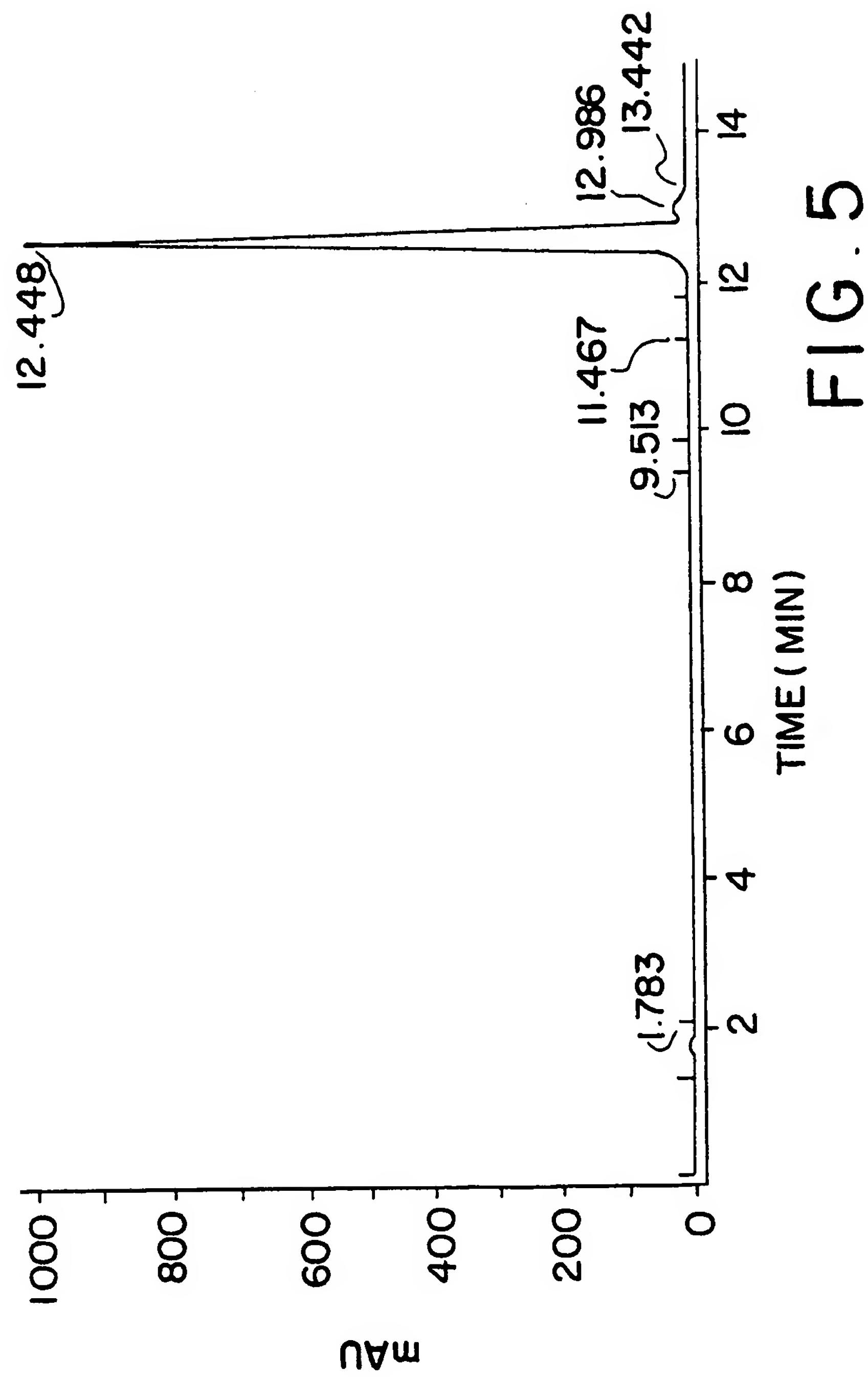


FIG. 5

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US92/02345

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C09B 29/01; 29/085, 29/095, 31/062; C12N 1/00, 1/14, 1/20

US CL :534/831,845; 435/243,252.35,254,262,263

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
X	US, A, 313,118 (STEBBINS, JR.) 03 March 1885, Note entire document.	1-23,28-30
X	US, A, 3,676,050 (JAMES) 11 July 1972, See table 1, Dye Nos. 1-116	1-23,28-30
X	US, A, 3,905,952 (SPECK) 16 September 1975, See claims 2 and 3.	1-23,28-30
X	Chemistry Letters, No. 1984 (YOSHIDA ET AL. I), "Rate Constants and Thermodynamic Parameters..." pages 703 to 706.	1-23,28-30
X	Journal of Physical Chemistry, Vol. 94, No. 10 issued 17 May 1990, (YOSHIDA ET AL. II), "Stability and structure of the Inclusion Complexes...", pages 4254-4259.	1-23,28-30
X	Colour Index, 3rd edition, Vol. 4, published 1971 by The Society of Dyers and colourist (Great Britain), see pages 4043 and 4087, especially C.I. Nos. 13010, 1305,13025,15970,15970, 15980 and 15985.	1-23,28-30

 Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

05 AUGUST 1992

Date of mailing of the international search report

08 SEP 1992

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Authorized officer

Fr. FIONA POWERS

1699 Ho Ngoc Ho
NGUYEN NGOC HO
INTERNATIONAL DIVISION

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US92/02345

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Index Chemicus, Vol. 29, No.9, Issue 237, abstract 95988, issued 27 May 1968 (Philadelphia, PA, U.S.A.) (GOODMAN ET AL.), "On the Synthesis and Identification of the Epinephrine Metabolite...".	1-23,28-30
X	Chemical Abstracts, Vol. 66, No. 17, Abstract 75812n issued 24 April 1967 (Columbus, Ohio, U.S.A.) Aspro-Nicholas, Ltd. "Analgetic and Anti-inflammatory Pharmaceuticals".	1-23,28-30
X	Chemical Abstracts, Vol. 81, No. 8, Abstract 38911h, issued 26 August 1974 (Columbus, Ohio, U.S.A.) (JAIN ET AL.), "Physicochemical Studies of the Cobalt (II) and Nickel (II) Chelates of Benzenesulfonate-Azo-p-Cresol".	1-23,28-30
X	Chemical Abstracts, Vol. 86, No. 4, Abstract 21734h, issued 24 January 1977 (Columbus, Ohio, U.S.A.) (MATUSIAK ET AL.), "Application of Arylsulfonic Acids for the Stepwise Separation..."	1-23,28-30
X	Chemical Abstracts, Vol. 90, No. 8, Abstract 62022a, issued 19 February 1979 (Columbus, Ohio, U.S.A.) (GHOLSE ET AL.), "Spectrophotometric Studies of Copper (II) and Palladium (II) with 3',5'-Dimethyl-2'-Hydroxyazobenzene-4-Sulfonic Acid (DMHAS)".	1-23,28-30
X	Chemical Abstracts Service Registry Handbook, published 1974 by the American Chemical Society (Columbus, ohio, U.S.A.) See registry nos. 2491-61-4, 2491-62-5,2491-63-6,2491-80-7 and 6287-12-3.	1-23,28-30
Y	US, A, 4,655,926 (CHANG ET AL.) 07 April 1987, Note entire document.	1-23,28-30
Y	US, A, 4,932,376 (FEEMAN) 13 January 1976, Note entire document.	1-23,28-30
X	Bulletin of Environmental Contamination & Toxicology, Vol. 17, No. 2, issued 1977, (Urushigawa et al.), "Chemico-biological Interactions in Biological Purification System II. biodegradation of Azocompounds by Activated Sludge" pages 214-218.	1,16
X, P	US, A, 5,091,089 (SHEN ET AL.) 25 February 1992, Note entire document.	1-23,28-30
A	Applied and Environmental Microbiology, Vol. 46, No.1, issued July 1983, (LEATHAM ET AL.), "Degradation of Phenolic Compounds and Ring Cleavage of Catechol by <u>Phanerochaete Chrysosporium</u> ", pages 191-197.	

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US92/02345

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

STN-FILE BIOSIS & CA, APS

Search (decoloriz? or biodeg? or degrad?) and (azo or monoazo or azadye# or azocompound#) and (phanerochaete or streptomyces or white(w)rot(w)fungus or fungus of fungi or microbe# or microorganism# or microbial)